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THE DESOXYRIBOSE NUCLEIC ACID CONTENT OF ANIMAL NUCLEI[1]

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THREE major chemical components of chromosomes have, so far, been described: two types of protein and desoxyribose nucleic acid (DNA). As one indication of the chemical identification of the gene, it is important to know how these components change with the diverse activities of the cell. The two chromosomal proteins have been shown to vary widely within the tissues of one animal. Mirsky and Ris (1949) have described a fivefold difference between the "residual protein" of calf thymus and that of calf liver. Miescher (1897) and Mirsky and Pollister (1943) have shown that during sperm formation in the salmon the histone disappears completely, to be replaced by protamine. In contrast to the proteins, however, evidence has recently been presented that DNA is considerably less variable in amount. Boivin, Vendrely, and Vendrely (1948) and Vendrely and Vendrely (1948, 1949) have published analyses of nuclear suspensions of several beef tissues. The number of nuclei analyzed was estimated by suspension counts and the amount of DNA per nucleus computed. These authors have reported that the DNA content of individual beef liver, thymus, kidney, and pancreas nuclei is approximately the same and that very nearly half this amount occurs in sperm. They have suggested that the amount of DNA per nucleus is probably constant in all the nuclei of any one species. More recent data presented by Mirsky and Ris (1949), obtained by similar methods, also indicate that DNA is approximately constant within the nuclei of one species. They also studied beef tissues, finding, however, that nuclei of liver, thymus, lymph node, and kidney averaged considerably more than twice that of the sperm. In a few species of fish, amphibians, reptiles, and birds the sperm contained more nearly half that found in liver and erythrocyte nuclei.

Not all quantitative studies on DNA have shown this indication of constancy per nucleus. Evidence that DNA varies widely from tissue to tissue in the plant *Tradescantia* has recently been brought forward by Schrader and Leuchtenberger (1949). They studied root, leaf, bud, and

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meiotic stages, finding an approximately threefold difference in the amount of DNA per nucleus between root and meiotic prophase nuclei, as shown by protometric determinations of Feulgen-stained preparations.

Other workers have suggested that DNA is not constant even within the nuclei of one cell type but, rather, varies with the metabolic conditions of the cell. During development of the sea-urchin egg, Brachet (1944) reports that DNA content of nuclei, as determined by the Feulgen reaction, is low per nucleus in the early cleavage stages, becoming much higher in blastula and pluteus nuclei. Gothie and Tsatsaris (1939; see also Moricard, 1949) report that the mouse oöcyte nucleus in early stages is Feulgen-negative but shows a rapid synthesis of DNA immediately before the first polar body is extruded. Many cytologists (Darlington and La Cour, 1940; Koller, 1947; Darlington and Mather, 1949; Serra, 1949) believe that the changes in content of DNA per nucleus parallel the relative distinctness of chromosomes in cytological preparations, increasing in prophase and decreasing gradually in telophase to a low amount in interphase. Serra (1949) has also considered desoxyribose nucleotides as functioning in energy transfer during protein synthesis, being synthesized on the chromonemata and converted into ribonucleotides in the nucleoplasm.

Several authors have described changes in the amounts of DNA per nucleus in diseased or experimentally treated tissues. Darlington and La Cour (1940) have reported that cold treatment of *Trillium* plants produced nuclei "starved" of DNA, as evidenced by the appearance of chromosome regions only weakly Feulgen-positive, although Wilson and Boothroyd (1941) attribute this to differential coiling. Koller (1946) found similar regions in the meiotic sex chromosomes of the hamster to be influenced by diet, disease, and age of the animal. Stowell (1945*b*) has reported a significant increase of DNA in Feulgen-stained human liver carcinoma and adenocarcinoma over that found in normal tissues. He has also (1947) reported increased amounts of DNA in lymphocytes of patients with lymphoid leukemia.

It is obviously of considerable importance in understanding the role played by DNA in nuclear processes to decide on the correctness of the theory, expressed by Boivin, Vendrely, and Vendrely (1948), as to the constancy of DNA in all the cells of a species. So far, the data that would support this view have come almost entirely from gross analyses of nuclear suspensions. Such techniques can present only the average amount of DNA for large numbers of nuclei, and individual variations, if any, are lost. Cytological methods, whereby constituents are measured in individual cells or parts of cells, do not have this inadequacy. It is obvious that a clear picture of the quantitative behavior of DNA can best be obtained by analyses of single nuclei.

If discrepancies from the constancy rule exist—and the data mentioned above would indicate that they do—it is of interest to know where they occur and with what processes they are associated. It is not too naïve to assume that the material of which genes are made possesses remarkable properties of chemical stability and is precisely determined in the nuclei of organisms. To find whether or not DNA could fill the exacting quantitative requirements of a gene component was the purpose of this study.

MATERIAL AND METHODS

In the present study the absorption of Feulgen-stained nuclei was determined by a photometric apparatus similar to

that described by Pollister and Ris (1947) and by Pollister and Moses (1949). Since the measuring technique used here differed somewhat from that used by previous workers, it will be described in some detail.

PHOTOMETRIC TECHNIQUE

The apparatus used is basically very simple. Any good, optically aligned microscope can be used for measuring, and the photometric accessories are not difficult to build. The parts are shown diagrammatically in Figure 1. Parts of the light source are mounted on an optical bench for easy adjustment; they are the lamp, collimating lens, lamp diaphragm, and filter-holder. Parallel light from the lamp passes through a firmly fixed right-angle prism, into the condenser, up through the specimen on a standard microscope slide, and through the objective and ocular. In making a reading, the nucleus to be measured is centered in the microscope field. Then the photometric apparatus is moved over the microscope, and a light-tight connecting sleeve or bellows is adjusted between the ocular and phototube. The enlarged, projected image of the nucleus is seen again through a magnifying lens placed beside the phototube. This magnifier contains an ocular micrometer and is placed immediately above a diaphragm, which eliminates all the image except that area of the nucleus to be measured. While the observer is looking through the magnifier, the nucleus can be made concentric with the field diaphragm by small movements of the mechanical stage, and the nuclear size can be measured with the micrometer. The magnifier is then swung out and the phototube moved into its place over the diaphragm, the phototube being so aligned that the image as seen in the magnifier now falls entirely on the photosensitive cathode. The microammeter deflection is read, the nucleus is moved away, and a clear area of the slide is similarly measured.[2] Two readings, one through the nucleus and one through the background, are thus made for each measurement. The intensity of light passing through the nucleus (I_n) divided by the intensity from a clear area of the slide (I_o) gives the transmission (T): $T = I_n/I_o$.

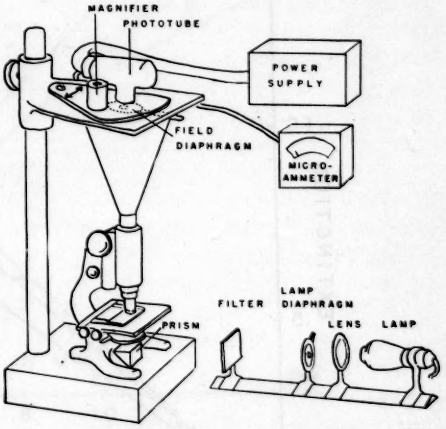

FIG. 1.—A simplified diagram of the photometric apparatus, showing all essential parts.

The transmission does not vary directly with the number of absorbing molecules but, rather, decreases logarithmically with a linear increase in the number of absorbing molecules. Thus, if one nucleus has a transmission of 10, a nucleus of the same size but with twice the con-

[2] All measurements were made with an RCA 931A electron multiplier phototube, operated on an a.c. power supply at 90 volts per dynode stage. Output leads were connected directly to a Weston microammeter with a maximum scale deflection of 10 microamperes. The scale could be read with reasonable accuracy to 1/100 of a microampere. A Western Union J-100 zirconium arc lamp provided intense and steady light for measurement. A Farrand interference filter with a peak transmission at 557 mμ and a half-band width of 15 mμ was used. Optics were a Zeiss N.A. 1.4 aplanatic condenser, Zeiss aprochromatic oil-immersion objective, 2 mm., N.A. 1.4, and a Spencer 10× high focal-point ocular.

centration of absorbing substance would have a transmission of 1, and with three times the concentration, of 0.1. The extinction (E) is defined as varying directly with the number of absorbing molecules, and consequently $E = 1/(\log T)$. Also since the number of molecules in the absorbing path depends upon the concentration of molecules (c) or the thickness (d) of the path, $E = kcd$, where k is a constant.

In obtaining the data presented here, the conditions of measurement were chosen so that, as nearly as possible, the extinction varied directly with the number of absorbing molecules in the light path. In photometric analyses of histological preparations, several factors tend to obscure this simple relationship, and conditions should obviously be selected to keep such factors at a minimum. Several possible sources of error are discussed below.

1. *Linearity of phototube response.*—The phototube should show a current output directly proportional to the intensity of incident light. Its photosensitive surface should also be uniform in sensitivity. The first criterion is well obeyed by the photomultiplier tube used, as can be seen by curve A in Figure 3. Neutral density filters held in the path of light decreased the galvanometer deflection in direct proportion to the filter transmission, as determined in a Beckman spectrophotometer. With regard to the sec-

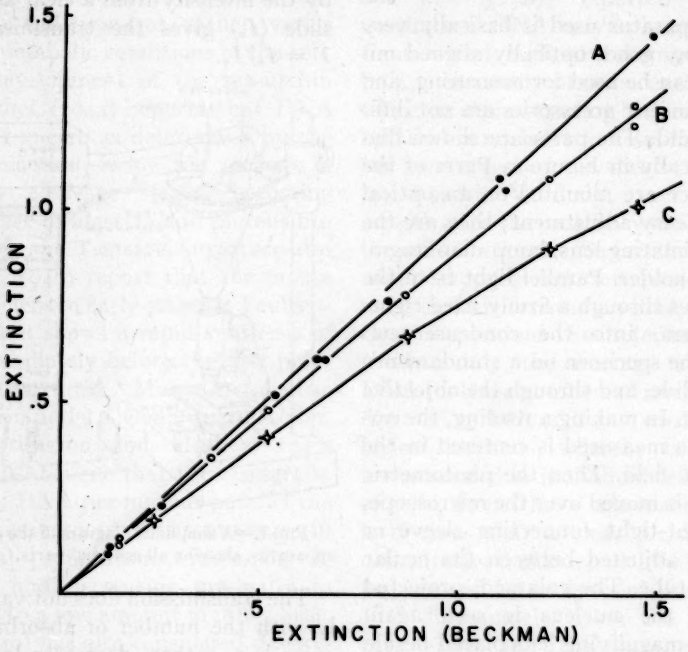

FIG. 2.—The relationship between extinctions, as determined with the microphotometer and with a Beckman spectrophotometer. Glass cuvettes were filled with solutions of formaldehyde-regenerated fuchsin and measured with both instruments. *A*, Microphotometer light source an AH_4 mercury lamp, with a Farrand interference filter tipped to allow only the 546 mμ line. *B*, Light source a zirconium J-100 arc lamp, with Farrand 557 mμ interference filter. *C*, Same zirconium-arc light source, with Wratten filter No. 58.

ond criterion, the cathode of electron multipler tubes is not uniformly photosensitive (Marshall *et al.*, 1948). However, the response is practically uniform within an area 4 mm. in diameter in the exact center of the cathode. Within this area, with a uniformly illuminated field of light, the galvanometer deflection is essentially proportional to the area illuminated, as can be seen in Figure 3, *B*. Outside the 4-mm. area the deflection is too low. It is thus important that the phototube be aligned so that the measured image falls exactly on the cathode center. In recent measurements a convex lens has been placed between the diaphragm and the phototube, reducing the size of the image focused on the cathode. In this way the linearity of phototube output can be extended to larger areas.

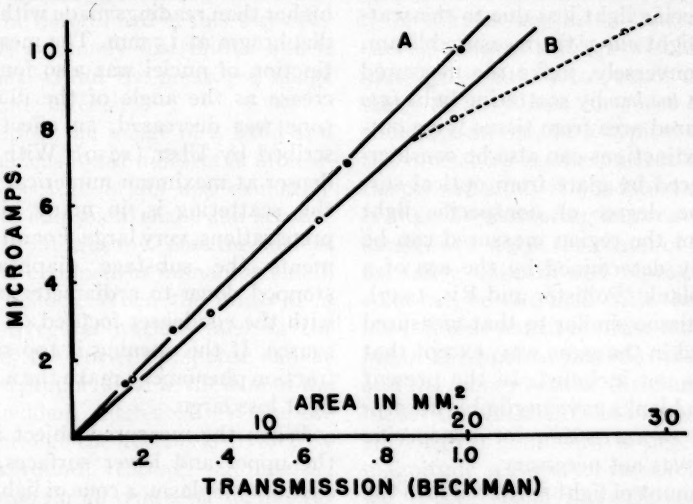

FIG. 3.—Linearity of current output of the 931A electron multiplier phototube. *A*, The output in microamperes, with differences in intensity of incident light produced with Bausch and Lomb neutral density filters, plotted against filter transmissions, as determined with a Beckman spectrophotometer. *B*, The output in microamperes, with differences in illuminated photosensitive area. A direct relationship is shown between the current output and the area of a spot of uniform intensity falling on the cathode. Since the most sensitive region of the cathode is approximately 4 mm. in width, areas wider than this show reduced output (*dotted line*).

2. *Color distribution of light source.*—Monochromatic light, passing through a dye at an absorbing wave length, is, if unaffected by other variables, extinguished in proportion to the number of dye molecules present. If impure light is used, the relation may cease to be linear. In the present study monochromatic light of the required intensity and stability was not obtainable, so that all measurements were made through an interference filter, which transmitted a certain amount of light of wave lengths above and below its peak. By measuring the same dye solution both in the apparatus and with monochromatic light in a Beckman spectrophotometer, the degree of distortion due to light impurity was determined. Curve *B* in Figure 2 shows this relationship for regenerated fuchsin, when measured with light of various degrees of purity. At the higher extinctions the error becomes considerable; consequently, all extinctions were corrected for this distortion (see Table 1).

3. *Illumination.*—Biological material

is usually full of colloidal particles, so that light passing through it is scattered, regardless of whether or not an absorbing dye is present. Also larger bodies, such as nuclei and nucleoli, when of different refractive index than the surrounding medium, reflect and refract light. This light-scattering can affect extinction readings in two ways. It can make them *too high* by nonspecific light loss due to the scattering of light *out of* the measured beam. It can, conversely, make the measured extinction *too low* by scattering light *into* the measured area from tissue lying outside it. Extinctions can also be considerably reduced by glare from optical surfaces. The degree of nonspecific light losses from the region measured can be adequately determined by the use of a suitable blank (Pollister and Ris, 1947), in which tissue similar to that measured is prepared in the same way, except that the dye is not included. In the present work such blanks gave negligible absorption, and so correction for nonspecific light loss was not necessary.

The amount of light scattered into the measured field is more difficult to determine. Two factors in the illuminating system are of major importance—the size of the field illuminated and the angle at which light converges on the specimen. The first factor is controlled largely by the lamp diaphragm, the second by the diaphragm in the substage condenser. To test the effect of the two diaphragms on extinction readings, small regions of spherical, Feulgen-stained nuclei were measured with various sizes of the two openings. The extinction for different points on the sphere was found to be that expected from the thickness only when the lamp and condenser diaphragm openings were small. When the diaphragms were wide open, transmission readings taken toward the nuclear margin tended to be proportionately too high. In the present study the lamp diaphragm was cut down to a diameter of 1 mm., with the result that the area illuminated was only a few diameters larger than that measured. This eliminated much of the glare and scattering from the surrounding tissue and increased the extinction readings considerably. In some cases extinctions were as much as 30 per cent higher than readings made with the lamp diaphragm at 15 mm. The measured extinction of nuclei was also found to increase as the angle of the illuminating cone was decreased, an effect also described by Uber (1939). With the condenser at maximum numerical aperture, this scattering is, in many histological preparations, very large. For all measurements the substage diaphragm was stopped down to a diameter of 4 mm., with the condenser focused on the light source. If the opening is too small, diffraction phenomena make the nonspecific light loss large.

When the measured object is flat on the upper and lower surfaces, as in a slice of cytoplasm, a cone of light, such as that from the condenser, obviously passes through more absorbing material than would a parallel beam. The optical path through such a solid is thus longer than the actual thickness. In the case of a sphere, however, as shown by Caspersson (1936), the mean lengths of the optical paths for converging and parallel light are very nearly identical. In the present study most measurements were made on spherical or ellipsoid nuclei, and the thickness of the absorbing substance was assumed to equal the mean length of path of parallel light through the structures measured.

When using extremely sensitive photomultiplier tubes, it is important that the photosensitive surface be shielded from all extraneous light. Room light must not leak into the system, and care must be

taken to avoid all internal reflections in the apparatus. A diaphragm placed in the microscope ocular helps to eliminate such reflections.

4. *Distributional errors.*—To determine, in arbitrary units, the total number of absorbing molecules, the measured extinction is multiplied by the area measured. Theoretically, the number of molecules can be determined in this way only if they are randomly distributed and if every point within the measured image has the same intensity.

In practice, the image of a fixed and stained nucleus is not uniform in intensity, both because of its approximately spherical or ellipsoidal shape and because of the fact that in many cases the nuclear material is clumped in numerous small, irregular masses. When the image of a typically irregular nucleus falls on the phototube, the current response can be considered proportional to the sum of the various incident intensities. When the reading through the nucleus is divided by the background reading, an *average* transmission for all points of the nuclear image is obtained. From this average transmission an extinction is computed, assumed to be the average extinction and thus proportional to the absorbing molecules within the nucleus. For most cytological material this assumption is not strictly correct (Fano, 1947). It can be shown that, while for every point in the image there can be found a transmission and a corresponding extinction, the *average* extinction is proportional to the *average* transmission only when all intensities in the image are the same. This is because the relation between extinction and transmission is complex, involving a logarithmic function. For any point (i) within the image $1/(\log T_i) = E_i$; but, in considering a large, heterogeneous image, $1/(\log \overline{T}) \neq \overline{E}$.

Measurements become more accurate, the more uniform the area measured. Since the uniformity desired is that in a plane *normal* to the light beam, uniformity of absorbing material in the direction *parallel* to the illuminating light beam is not necessary. Uniformity of image area can be increased by measuring a small region in the center of the nucleus rather than the whole structure. This practically eliminates the error caused by the curved surfaces through which the light must pass; but, if too small an area is taken, it may not, for most fixed and stained material, be a typical sample of the intensities in the nuclear image. In the present study it was found empirically that, by measuring an area with a diameter of 2–4 μ in the center of the nucleus, fairly uniform and reproducible results can be obtained with the fixatives used. (Some fixatives cause more clumping of the nuclear material than others; and the more irregular the clumping, the higher the variability in measurements.) Although it is difficult to determine the magnitude of the error caused by clumping, it is probable that differences in distribution of stained material from nucleus to nucleus, in the present study, resulted in an error of, at most, 15 per cent in the more unfavorable cases and was usually somewhat smaller. Therefore, although the unhomogeneous distribution of absorbing material may offer a large and indeterminate variable, the error can be reduced by using certain fixatives and by the measurement of a central area rather than of the whole nucleus.

In all the measurements presented here, an area of 4, 3, or 2 μ was measured through the center of the nucleus, depending on nuclear size. The radius of each nucleus, if circular in outline, was also measured, or the major and minor axes if the nucleus was ellipsoid. Size measurements were made as accurately as possible to the nearest 0.2 μ. The

image was visible in the magnifier enlarged two thousand times, imposed on an ocular micrometer, ruled in tenths of a millimeter, enlarged two times. Care was taken to see that all the nuclei measured were entire, as seen by focusing on material above and below their level, and were not overlapped by surrounding nuclei. Sections were cut at varying thicknesses (from 6 to 20 μ); thick enough so that whole nuclei were frequently obtained and yet not thick enough to cause too much overlapping of nuclei.

Where care is taken in selecting entire nuclei, not overlain, and when nuclear size is carefully measured, reproducible results can be obtained. The degree of reproducibility depends on the material. When nuclei are regular in outline, the distribution of stained material fairly homogeneous, the size large and thus the volumes accurately computable, standard deviations of about 6 per cent have been obtained for a series of measurements, as shown in the formalin-fixed frog material discussed below. When nuclei are irregular in outline and the chromatin is unevenly distributed, the variability becomes much greater. The mouse Sertoli cells measured showed a standard deviation of 17 per cent. These nuclei were flattened and irregular, with a few intensely staining granules.

When the central area of a spherical nucleus is measured, multiplying the obtained extinction by the central area gives, in arbitrary units, a measure of the molecules within a cylinder cut through the nucleus. The cylinder obviously contains only a fraction of the total volume of the nucleus; so, to obtain a value for the total nuclear volume, the extinction must be divided by this fraction. The volume of a central cylindrical region cut from the center of a sphere is given by $V = \frac{4}{3}\pi(R^3 - H^3)$, where R is the radius of the sphere and H is half the height of the cylinder. Also $H = \sqrt{R^2 - C^2}$, where C is the radius of the cylinder. For each nucleus measured, C and R were determined, and the extinction was divided by that fraction (F) of the total nuclear volume included in the cylinder, where

$$F = \frac{R^3 - (R^2 - C^2)^{3/2}}{R^3}.$$

A table was prepared, giving F for different values of R and C. This made calculation of the total absorbing substance per nucleus rapid and simple. Nuclear volumes were treated as spheres or prolate spheroids in most cases. For spheroids, except when markedly aspherical, the major and minor axes were averaged, and this mean was assumed to be the radius of a sphere. This usually involved only a small error and saved much calculation. An example of the raw data and their treatment is given in Table 1. In the first column are listed the nuclear diameters or the longest and shortest dimensions if the nucleus is aspherical. The second column gives the galvanometer deflections for an area 4, 3, or 2 μ in diameter through the center of the nucleus (I_n); and the third column gives the background intensity (I_o) taken immediately after the I_n reading. The fourth column gives the transmission (I_n/I_o), and the fifth column the extinction ($\log_{10}[I_o/I_n]$). Since the light used was not monochromatic, all extinctions were corrected by the amount graphed in Figure 2*B*. The sixth column contains the corrected extinctions. The seventh column lists these extinctions multiplied by the squared radius of the measured area and divided by the fraction of the total volume measured (C^2/F).[3]

[3] The method of measuring a central area in the nucleus is, it is felt, considerably more flexible and accurate than the procedure used by Ris and Mirsky (1949*b*). These authors measured an area circum-

TABLE 1

SAMPLE RAW DATA, SHOWING THE CALCULATIONS MADE IN OBTAINING THE ARBITRARY UNITS GIVEN IN SUBSEQUENT TABLES

Nuclear Size (μ)	I_n	I_o	Transmission	Extinction	Corrected Extinction	$E(C^2/F)$
	a) Neurones from 16-day mouse spinal cord; fixation 50 per cent formalin; area measured 4 μ in diameter					
10.0×8.0	35.8	59.5	60.2	0.220	0.234	3.34
11.0×8.2	38.0	58.0	65.5	0.183	0.193	3.10
8.4×5.2	26.0	58.8	44.2	0.354	0.380	3.21
13.6×8.0	42.8	57.3	74.7	0.127	0.130	2.62
9.2×8.0	34.2	57.3	59.7	0.225	0.239	3.25
9.2×7.0	35.0	57.5	60.8	0.216	0.229	2.68
6.6×4.4	15.8	53.0	29.8	0.525	0.576	3.41
9.8×8.0	33.0	57.5	57.3	0.242	0.258	3.61
7.6×6.6	30.0	57.5	52.2	0.282	0.303	2.78
8.0×7.2	30.4	57.5	52.8	0.278	0.299	3.11
	b) Nuclei from adult mouse pancreas; fixation 50 per cent formalin; area measured 4 μ in diameter					
4.6×4.0	10.7	53.5	20.0	0.699	0.783	3.29
6.4×5.0	19.5	58.0	33.6	0.474	0.520	3.26
8.2×5.4	12.8	64.2	19.9	0.701	0.785	6.64
6.4×5.6	23.7	68.0	34.8	0.459	0.500	3.41
7.4×5.0	22.7	64.0	35.5	0.450	0.490	3.06
8.0×7.0	16.5	67.2	24.5	0.611	0.679	6.88
7.2×6.8	14.3	65.0	22.0	0.657	0.732	6.48
5.0×4.0	14.9	65.5	22.8	0.642	0.716	3.17
9.0×6.4	5.7	68.0	8.4	1.07	1.23	13.1
4.8×4.0	14.1	62.5	22.5	0.648	0.722	3.11
	c) Nuclei from liver of 11-day mouse embryo; fixation Carnoy acetic alcohol; area measured 4 μ in diameter					
8.2×7.6	27.5	60.5	45.4	0.342	0.369	4.12
8.4×6.6	26.8	55.5	48.3	0.316	0.337	3.42
9.4×6.6	23.2	56.1	41.4	0.382	0.412	4.72
7.0×6.6	22.2	56.5	39.3	0.405	0.439	3.72
7.8×6.2	25.2	57.3	44.0	0.356	0.380	3.38
8.2×5.4	24.8	54.0	45.9	0.338	0.361	3.05
7.6×5.2	23.5	54.5	43.1	0.366	0.393	3.00
11.2×6.8	36.2	53.2	67.9	0.167	0.175	2.50
6.8	27.2	55.3	49.2	0.308	0.328	2.77
8.4×7.0	23.0	56.0	41.1	0.386	0.416	4.44
	d) Frog spermatid nuclei; formalin fixation; area measured 3 μ in diameter or 2 μ where marked with asterisk					
3.4	3.2	38.0	8.4	1.08	1.23	3.08
2.8*	0.7	16.4	4.3	1.37	1.67	2.55
2.8*	0.6	15.5	3.9	1.41	1.71	2.60
3.0*	0.7	15.5	4.5	1.35	1.65	2.82
3.8×3.4	3.2	32.5	9.8	1.01	1.14	3.08
3.6×3.0	3.0	34.2	8.8	1.05	1.18	2.86
3.4×3.0	3.0	39.2	7.7	1.11	1.27	2.98
3.8×3.4	3.8	38.2	9.9	1.00	1.15	3.12
3.4	4.0	38.5	10.4	0.983	1.12	2.80
3.4×3.2	3.2	34.0	9.4	1.03	1.18	2.86

5. *Molecular orientation.*—One special type of distributional error that may arise in photometric studies has recently been discussed by Commoner (1949) and Pollister and Swift (1950). Where absorbing molecules are oriented in a crystal-like structure, all the absorbing resonances may occur in specific directions rather than at random, giving rise to dichroism. Thus, under polarized light, different extinctions would be obtained for different angles of the polarizer, and, under nonpolarized light, extinctions would tend to be lower than those given by unoriented molecules. Commoner's discussion primarily considered the possible occurrence of such errors in measurements with ultraviolet light. He pointed out, however, that many biological structures contain oriented molecules and that in some instances the dyes combined with aligned molecules may also be oriented. The possibility of error caused by this effect should, of course, be considered in measuring any biological material. However, as shown by Pollister and Swift, the sweeping contention of Commoner that orientation tends to invalidate biological photometric analysis as a whole is unjustified.

Dichroism has been described by Caspersson (1940) for the ultraviolet absorption of living grasshopper (*Gomphocerus*) sperm. In the present study it was not found for any Feulgen-stained material, including grasshopper (*Chortophaga*) sperm and mouse liver nuclei, and thus does not affect the present data. Data of the type given here could not be obtained, were the effect of more than negligible importance in the material studied.

FIXATION AND STAINING

In the course of the study, tissues were measured from several animal species. Laboratory mice[4] of both sexes were used, at the following ages: 11-day fetus, 1-, 10-, 16-, and 30-day-old young, and adults; in all, twelve different mouse tissues were studied. An adult male Wistar rat was used for the spermatid measurements shown in Figure 5. Six different frog tissues were measured, all from an adult male *Rana pipiens*. Data presented in Figure 4, showing Lambert's law, were taken from liver nuclei of an adult male *Ambystoma maculatum*. Liver, pronephros, and erythrocyte nuclei were measured from a newly hatched (stage 46) larva of *A. opacum*. Spermatid and Malpighian tubule nuclei were measured from an adult male acridid grasshopper (*Dissosteira carolina*). Except where stated to the contrary, all measurements were made on interphase nuclei.

All material studied was fixed in formalin of a concentration of 30 or 50 per cent (i.e., the stock 40 per cent formaldehyde solution diluted with 2 parts, or 1 part, of water), except for the mouse embryo and *Ambystoma* larva, which were fixed in Carnoy's acetic alcohol (3 parts of absolute alcohol to 1 part of glacial acetic acid). Tissues were removed from

scribing the entire nucleus, of necessity letting in background light where nuclei were not precisely round. This method is doubtless largely responsible for the difference of over 30 per cent found by them between the measured values of liver and erythrocyte nuclei, a discrepancy which Ris and Mirsky seem wrongly to have attributed entirely to different distribution of chromatin. These authors feel that, where nuclei of different types are to be compared, they must first be mashed and smeared on slides to create uniform staining, a procedure that would considerably limit the application of photometry to tissues and which, as indicated in the present work, is fortunately unjustified. A homogeneous distribution of the absorbing molecules in the area measured is, of course, desirable, as mentioned above; but most vertebrate tissues are measurable in sectioned material with the techniques here described, and distributional errors are usually small.

[4] From the genetic stocks of Dr. L. C. Dunn. The writer is indebted to Dr. Dunn and to Dr. S. Gluecksohn-Schoenheimer for their kindness in supplying this material.

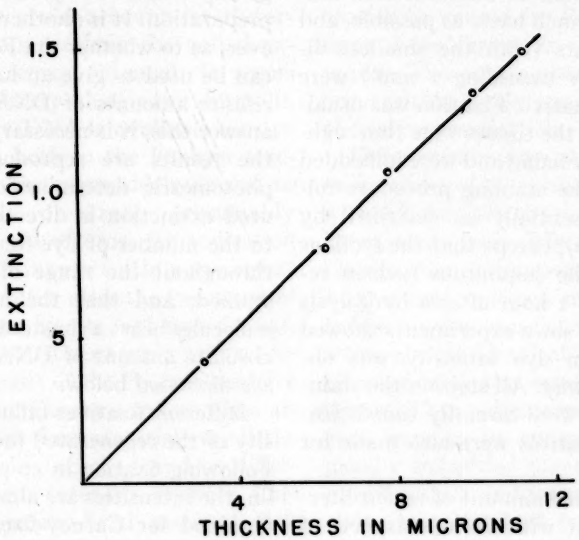

FIG. 4.—The relationship between extinction and thickness of the absorbing layer (Lambert's law) as shown by Feulgen-stained *Ambystoma* liver nuclei cut at various thicknesses. (Cf. Pollister and Swift, 1950.)

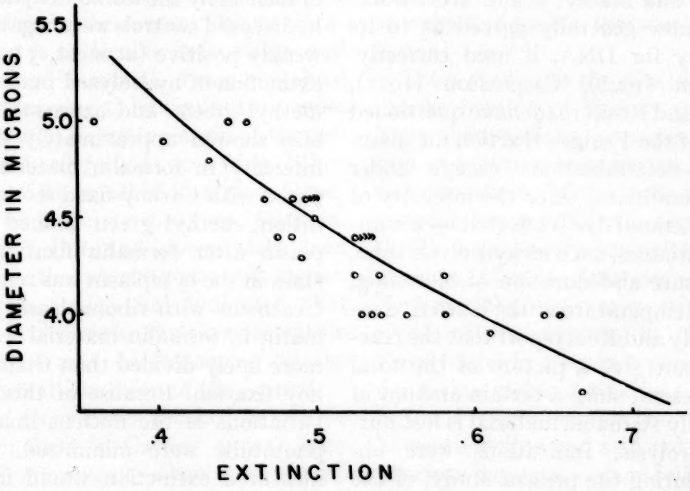

FIG. 5.—The relation between size and extinction of 32 Feulgen-stained rat spermatid nuclei. The solid line shows the theoretical relationship between nuclear diameter and extinction, where the number of absorbing molecules is constant. (Cf. Pollister and Swift, 1950.)

the freshly killed or anaesthetized animals with as much haste as possible, and small fragments (with the smallest dimension rarely exceeding 1 mm.) were cut into the fixative. Fixation was usually for 3 hours; the tissues were thoroughly washed after fixing and were imbedded in paraffin. The staining procedure followed was essentially as described by Stowell (1945*a*), except that the sections were left in the sulphurous fuchsin reagent for only 1 hour after a hydrolysis of 14 minutes, since experiments showed that maximum dye intensity was obtained in this way. All steps in the staining procedure were carefully timed. Unhydrolyzed controls were also made for all tissues.

A considerable amount of recent literature has dealt with various aspects of the Feulgen reaction (Stowell, 1945*a*, 1946; Di Stefano, 1948; Ely and Ross, 1949; Stedman and Stedman, 1947; Overend and Stacey, 1949). Most workers are now generally agreed as to its specificity for DNA, if used correctly. Widström (1928), Caspersson (1932), and Ely and Ross (1949) have questioned the use of the Feulgen reaction for quantitative determinations except under special conditions, since the intensity of the regenerated dye is affected by a number of variables, such as hydrolysis time, temperature and duration of bleaching, pH and temperature of the leuco dye solution. Ely and Ross report that the reaction cannot give a picture of the total DNA present, since a certain amount of potentially stainable material is lost during hydrolysis. Indications were obtained, during the present study, of the variability of the reaction with slight changes in the staining procedure. It is doubtful to what extent one is justified in making computations of the absolute amounts of DNA within a nucleus on the basis of the number of regenerated Feulgen-dye molecules present in a stained preparation. It is another question, however, as to whether the Feulgen reaction can be used to give an indication of the *relative* amounts of DNA in nuclei. To answer this, it is necessary to know that the results are reproducible, that, in photometric determinations, the measured extinction is directly proportional to the number of dye molecules present throughout the range of concentration studied, and that the number of dye molecules bears a constant relation to the absolute amount of DNA. These points are discussed below.

Different fixatives influence the intensity of the regenerated fuchsin obtained. Following fixation in 50 per cent formalin, the intensities are almost twice those obtained for Carnoy-fixed material for the same type of nuclei, although ratios between nuclear classes remained the same. This is not caused by regeneration of fuchsin by the formaldehyde, since unhydrolyzed controls were negative or very weakly positive (at most, 5 per cent the extinction of hydrolyzed material). Both methyl green– and azure-stained nuclei also showed approximately double the intensity in formalin material as compared with Carnoy-fixed sections. In addition, methyl green stained the cytoplasm after formalin fixation, but all stain in the cytoplasm was removable by treatment with ribonuclease. The chromatin in formalin material was usually more finely divided than that after Carnoy fixation. Because of this, intensity variations of the nucleus image on the phototube were minimized. Since the measured extinction would increase as the image intensities approached uniformity, other things being equal, nuclei with the dye evenly distributed should give slightly higher extinctions than nuclei with clumped, irregular chromatin. It seems unlikely, however, that the dif-

ference in distribution obtained with the two fixatives could account for distortions at all approaching the actual extinction differences obtained. It seems much more likely that, after formalin fixation, more DNA is available for dye combination, both to the Feulgen reaction through the sugar aldehyde and to methyl green or azure, presumably through the phosphorus. An indication that the DNA may occur in a somewhat different condition after the two types of fixation is seen by the fact that desoxyribonuclease treatment sufficient to remove all or most of the Feulgen-positive material from Carnoy-fixed sections affected formalin-fixed material very little. Ris and Mirsky (1949*b*) found no difference in Feulgen intensity between Carnoy- and formalin-fixed material, but lower concentrations of formalin were used.

As all cytologists know, tissues often stain differently at the periphery of the section than in the center. This penetration effect is noticeable in formalin- and Carnoy-fixed material. Feulgen-stained nuclei were found to be significantly more intense along the outer margin of the section, in a border about 50 μ wide. When liver tissue was mashed in a glass homogenizer in 30 per cent sucrose solution, spread on a slide, and immediately fixed in Carnoy, the intensity was still higher. Lagerstedt (1949) has indicated that changes in chromatin distribution within the nucleus may be influenced by the physiological condition of the animal.

Nuclei from tissue mashed in sucrose and then fixed in Carnoy appear completely homogeneous when stained with Feulgen dye, except for central clear areas occupied by nucleoli. This same sort of homogeneity is characteristic of living nuclei as seen under phase contrast or normal illumination. It is thus obvious that, in many cases, the granular appearance found commonly after cytological fixation is caused by localized contractions of the chromatin (Ris and Mirsky, 1949*a*). This contraction can apparently occur within the extremely brief interval of fixative penetration.

Differences in intensity were found between slides of the same material stained with different preparations of Feulgen reagent made from different lots of basic fuchsin. Also, extinction differences up to 15 per cent between means of nuclear samples were occasionally found, even when almost identical procedures were followed with the same tissues and reagents. Thus, when several different tissues were to be compared, they were usually imbedded together in the same paraffin block and mounted on the same slide. As a standard for comparison, in some cases, a section of a tissue studied previously, usually mouse liver, was placed on a slide together with unknown material, and both were then measured, as suggested by Ris (personal communication).

These studies on the effects of fixatives can be considered as only preliminary. They demonstrate, however, that fixation and treatment of tissues before fixation can affect the intensity of the Feulgen reaction as well as the distribution of the Feulgen-positive material within the nucleus. Also, at least in some cases, Feulgen intensity varies with the position of the nucleus in the section, those nuclei in a narrow peripheral border giving higher extinctions.

The Feulgen reaction, however, could be reproducible and still not present an accurate measure, even in relative terms, of changes in DNA in tissues. Widström (1928) and Caspersson (1932) have shown that, *in vitro*, DNA will give different intensities when different amounts of proteins are added. Lessler (1948) studied the color produced by agar in which vari-

ous concentrations of DNA were included. He concluded that the reaction was quantitative for the DNA concentrations normally found in tissues, but estimates of dye intensity were made only by eye.

Obviously, it is more important to know the relationship between DNA and dye intensity in cytological preparations than to rely on *in vitro* experiments with nuclear extracts. To show that the dye intensity, as measured photometrically, varies as the thickness of the absorbing layer (Lambert's law), *Ambystoma* liver nuclei were cut at various thicknesses, and their extinctions were measured. The results (Fig. 4) show the expected linear relationship for an absorbing material with an approximately uniform distribution of dye molecules throughout its volume.

A linear relationship must also be shown between dye intensity and concentration. When measuring a series of Feulgen-stained nuclei of any one type, one is struck by the fact that smaller nuclei are darker and larger nuclei lighter. When corrections for nuclear volume are made, it is obvious that approximately the same number of dye molecules exists in each nucleus, regardless of nuclear size. In Figure 5 the extinction values for 33 rat spermatid nuclei are plotted against their diameters. The solid line shows the theoretical relationship between diameter and extinction if the number of absorbing molecules remains constant. The measured values group around this line.

These data can be interpreted in two ways. Either the Feulgen reaction gives an accurate estimate of the number of dye molecules within the nuclei for various concentrations and thicknesses, or the Feulgen reaction is not linear with concentration, but this distortion is exactly counteracted by some other variable. The relationship between size and extinction has been found to hold for over three thousand nuclei, ranging in extinctions from 0.1 to 1.5. It is difficult to conceive of any hypothesis to explain these results other than that the Feulgen reaction, as used here, gives an accurate relative measure of the DNA in nuclei.

Several previous workers have chosen to convert the measured extinction values into estimates of the amount of Feulgen dye or the amount of DNA per nucleus (Di Stefano, 1948; Pollister and Leuchtenberger, 1949; Leuchtenberger, 1950). To determine the number of molecules of Feulgen dye per nucleus, one must know the extinction, per molecule, of the Feulgen-DNA complex. This is difficult to determine under the conditions in which the compound exists in various histological preparations, although it can be approximated by *in vitro* regeneration (Di Stefano, 1948). In addition, variations in extinction are caused by variations in the conditions of illumination, and it is difficult to determine which conditions are correct. Lastly, as mentioned above, variations in the fixation and staining procedures produce variations in the intensity of the reaction, and probably some potentially stainable material is lost during hydrolysis. Clearly, photometric studies of Feulgen-stained nuclei can be made to give *absolute* amounts of DNA only when certain tissues, whose DNA content has been correctly determined by other means, are used as standards of comparison. Comparisons have recently been made by Ris and Mirsky (1949*b*) between photometric determinations and chemical analyses. Unfortunately, gross chemical determinations of DNA made by different workers on similar material are not in good agreement (Zittle and Odell, 1941; Boivin, Vendrely, and Vendrely, 1948; Mirsky and Ris, 1949). In the present study all data are presented in arbitrary

units of Feulgen dye per nucleus. The measured extinctions have merely been multiplied by the squared radius of the measured area (C^2), and divided by the fraction (F) of the total volume included in the central measured area, as shown in Table 1.

RESULTS

The photometric data show a remarkable unity in the computed amounts of regenerated fuchsin per nucleus in the various tissues measured (Swift, 1949). The constancy of total dye holds for mouse tissues over a size variation of almost twenty times, from the smallest embryo normoblast measured (24 μ^3) to the largest motor neurone (460 μ^3). As stated above, it is difficult to imagine how this agreement between values for a variety of nuclear types in an organism could be explained on any grounds other than that the Feulgen intensity, as determined photometrically, gives an accurate relative measure of the DNA in nuclei.

MOUSE TISSUES

Interphase nuclei were measured from the sex cells and 10 somatic tissues of young and adult mice. All material was fixed in 50 per cent formalin. The results from somatic cells are summarized in Table 2 and from sex cells in Table 3. These tables show the average amounts of DNA per nucleus, expressed in arbitrary units. All the mouse nuclei measured from differentiated tissues gave approximately the same values, with the exception of certain nuclei in the liver, pancreas, thymus, lymphocytes from blood, Sertoli cells, and various stages of sperm formation. In these tissues some of the nuclei measured were twice or four times the common value, and the spermatids were half the common value. In the present paper those nuclei falling into the commonest group are placed in Class I, those with values twice as large in Class II, and those with values four times as large in Class III. All the nuclei measured in nondividing tissues fell into these

TABLE 2

AVERAGE AMOUNTS OF DNA PER NUCLEUS IN FORMALIN-FIXED SOMATIC TISSUES OF YOUNG AND ADULT MICE, AS OBTAINED BY PHOTOMETRIC DETERMINATIONS OF FEULGEN PREPARATIONS*

Cell Type		DNA (Feulgen)	Standard Error	No. of Measurements
Liver	Class I	3.34	.05	21
	Class II	6.77	.07	52
	Class III	13.2	.25	12
Pancreas	Class I	3.10	.06	20
	Class II	6.36	.09	15
	Class III	12.4	.....	5
Thymus	Class I	3.28	.06	33
	Class II	6.17	.18	21
Lymphocytes	Class I	3.20	.08	19
	Class II	6.00	.22	9
Sertoli cells	Class I	3.00	.12	18
	Class II	6.40	.26	7
Kidney tubule		3.14	.04	30
Small intestine epithelium		2.97	.04	20
Spleen		3.12	.04	33
Neurones (spinal cord)		3.14	.07	20
Interstitial cells (testis)		3.05	.08	20

* Averages are expressed in arbitrary units (see Table 1 and text).

TABLE 3

AVERAGE AMOUNTS OF DNA PER NUCLEUS IN FORMALIN-FIXED DEVELOPING SEX CELLS FROM THE MOUSE TESTIS, AS OBTAINED BY PHOTOMETRIC DETERMINATIONS OF FEULGEN PREPARATIONS*

Cell Type		DNA (Feulgen)	Standard Error	No. of Measurements
Premeiotic	Class I	3.28	.07	33
	Class II	5.96	.07	78
Spermatocyte I		6.28	.07	41
Spermatocyte II		3.35	.04	30
Spermatid		1.68	.02	28

* Averages are expressed in arbitrary units (see Table 1 and text).

three classes, except the spermatids with half the Class I value. These classes, as they appear in Feulgen-stained pancreas, are shown in Plate II, Fig. *C*.

Sections of liver tissue, all from the same block, were placed on most of the slides measured as a standard for comparison. One slide, containing several of the tissues listed in Table 2, gave values about 12 per cent higher than normal; but, since the standard liver section was also higher, it is apparent that the Feulgen reaction was, for some reason, more intense on this slide. Values from this slide were corrected downward according to the ratio of liver measurements. Spleen and thymus sections were difficult to measure because of overlapping nuclei. A few cells from these tissues were measured in sectioned material, but most readings were taken from material mashed in sucrose and fixed in formalin after it was spread on slides. These preparations gave values similar to the sectioned material. Small differences between the means of different types of nuclei listed in Table 2 are, in some cases, statistically significant. These are probably distributional errors, caused by differences in chromatin arrangement, roughly constant within one cell type but differing somewhat from tissue to tissue.

Age and class frequencies.—Liver nuclei were measured from 10-day-old and adult mice. The distribution of the values obtained is shown graphically in Figure 6. Although no definite attempt was made to pick nuclei completely at random, the number of measurements falling into each class gives an approximate indication of the class frequencies. In the young mouse, Class I nuclei were common, and none of Class III were found. In the adult, Class II was often as common as Class I and occasionally more so. In the adult tissue no intermediate values were found between classes, but in the 10-day liver a few measurements fell midway between Classes I and II. These intermediate values, as discussed below, are caused by the building-up of DNA prior to cell division.

Formation of sex cells.—Testes were studied from adult and young mice 1, 10, 16, and 30 days old. In the 1-day testes the first premeiotic cells were in the tubules. These were of two types, falling into Classes I and II, as can be seen in photographs *A*, *B*, and *C* of Plate I. The Class I premeiotic nuclei were more common. Both nuclei have a dense and fairly uniformly distributed chromatin in formalin preparations and, except for size, are similar morphologically. A few very large and pale nuclei were found in the 1-day and 10-day testes. Since these were always cut or overlapped, they could not be measured.

In the 16-day testis the Class II premeiotic nuclei were very abundant, and many were enlarging slightly in size to resemble early leptotenes (Pl. I, Fig. *D*). An occasional tubule was found, containing nuclei falling into Class III (Pl. I, Fig *F*), resembling enlarged nuclei of Class II. These were rare and were found in only one 16-day testis. A few tubules in the 16-day testis contained Class I nuclei. In the adult, Class I nuclei were uncommon but could occasionally be seen against the tubule wall (Pl. II, Fig. *B*). The commonest premeiotic cells in the adult were those of Class II, which commonly lined the tubules (Pl. II, Fig. *A*).

Typical meiotic prophase stages were common in the 16-day testis. All those that were measured, from leptotene to early diakinesis, gave values approximately equal to the Class II premeiotic nuclei. Thus there was no indication of an increase in DNA during the meiotic prophase. In the 30-day testis secondary spermatocytes were common and gave

values falling into Class I. Spermatid nuclei, measured in the 30-day and adult testes, gave values averaging half that of the secondary spermatocytes. These data are summarized in Table 3 and are shown graphically in Figure 7.

It is of considerable importance in determining the seriation of the premeiotic stages to know which cell types are dividing. Gonial division figures were infrequent in the preparations studied, but some were found at all ages. A gonial prophase and metaphase, from a 1-day testis, are shown in Plate I, Fig *B*. Gonial metaphase plates, where chromosome number was countable, showed the normal diploid complement. As will be shown in the following paragraphs, DNA in dividing embryonic mouse liver and the kidney of *Ambystoma* larvae increases in amount for division sometime before the visible onset of prophase. If this is also true of dividing spermatogonia, measurements on either early prophase

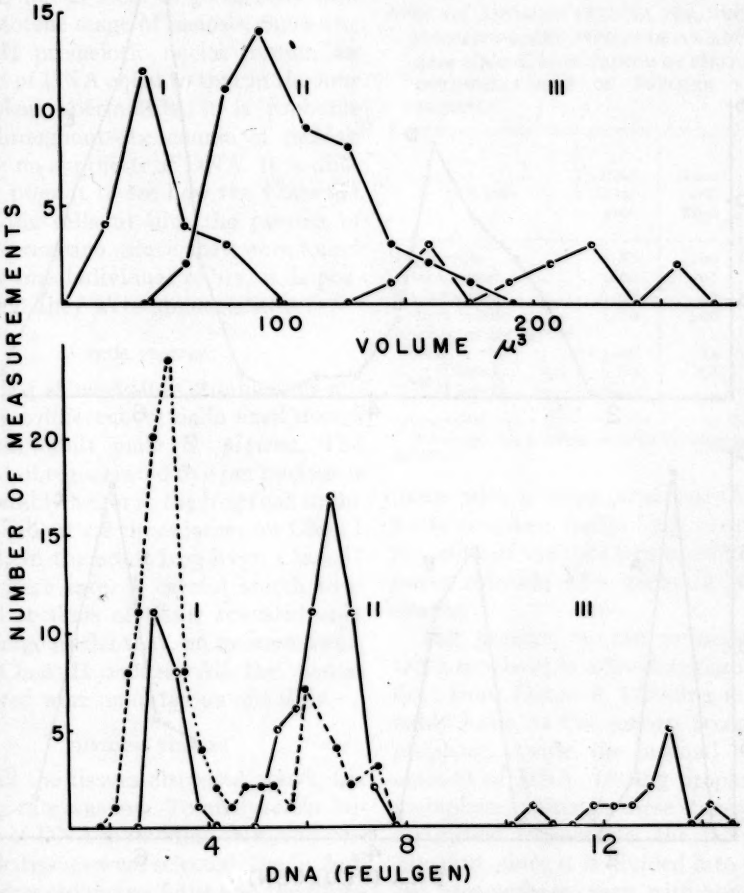

FIG. 6.—*Lower graph:* Amounts of DNA, in arbitrary units, in individual mouse liver nuclei as indicated by the Feulgen reaction; *dotted line*, 10-day mouse; *solid line*, adult. *Upper graph:* Volume relations of the same nuclei shown by solid lines below, as segregated by their DNA content.

or late telophase nuclei would give an indication as to whether Class I or Class II cells or both are responsible for the division figures seen. Only 10 of the division figures found were measurable, 6 prophases and 4 telophases. All gave values obtainable only from Class I divisions, i.e., prophase values falling in Class II and telophase values in Class I. Several other prophase or telophase nuclei were found but were unmeasurable because they were overlapped or cut. By visual inspection these seemed similar to those measured.

For a complete analysis of the changes in DNA during sex-cell formation in the male, more information is required than that presented here. At present it seems

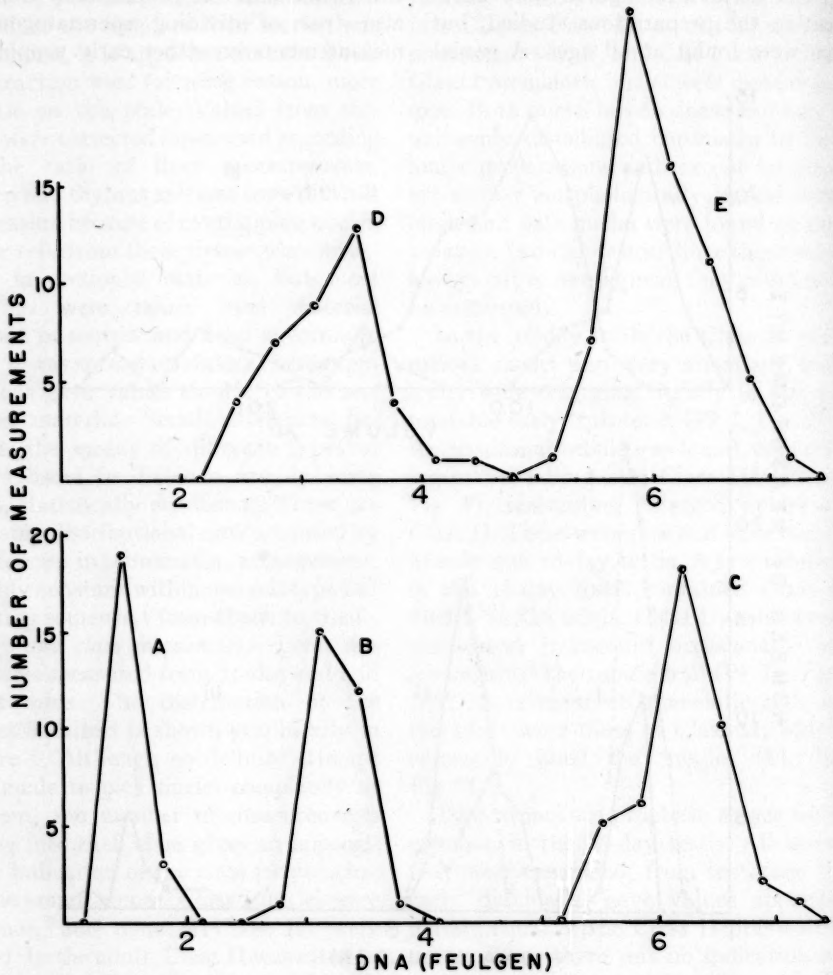

FIG. 7.—The amounts of DNA, in arbitrary units, in individual nuclei from the mouse testis. *Upper graph:* Classes I and II premeiotic nuclei. *Lower graph: A*, spermatids; *B*, secondary spermatocytes; *C*, primary spermatocytes.

most likely that Class I premeiotic cells are formed first and that these either divide mitotically to form more cells of Class I or build up DNA without dividing to form Class II nuclei. If the process occurs in this way, the change from Class I to Class II must be comparatively rapid, since, as shown in Figure 6, few intermediates between the classes were found. The few intermediates could also be interpreted as stages in DNA increase previous to the division of Class I cells. Class II nuclei seem to go directly into the leptotene stage of meiosis. Since the Class II premeiotic nuclei contain an amount of DNA equal to that in the four descendent spermatids, it is probable that throughout the course of meiosis there is no synthesis of DNA. It is difficult at present to see how the Class III premeiotic cells fit into the process of sperm formation. Since they were found in only one individual of six, it is possible that they were abnormalities.

FROG TISSUES

Table 4 shows values obtained for nuclei of six different formalin-fixed tissues from an adult male *R. pipiens*. The amount of regenerated dye per nucleus is considerably larger in the frog than in the mouse (about 1.8 times larger for Class I nuclei). In the adult frog liver, Class II nuclei were rare. A careful search over several sections of tissue revealed only three large nuclei that, on measurement, gave Class II values. All the tissues measured were mounted on one slide.

DIVIDING TISSUES

In all the tissues discussed above, the mitotic rate was low. To analyze the behavior of DNA in dividing cells, two embryonic tissues were selected: the liver of an 11-day-old mouse fetus and the forming pronephros of a newly hatched (stage 46) larva of *A. opacum*. Material was Carnoy-fixed. The high mitotic rate in the *Ambystoma* pronephros can be seen in Plate II, Fig. *D*. The distribution curve for interphase nuclei in this organ is shown in Figure 8, *C*. The values are distributed over a broad curve, most measurements falling at the lower end. Mitotic pronephros nuclei are shown by the dotted lines in Figure 8, *A*, as compared with the Class I and Class II nuclei from *Ambystoma* liver (solid lines), a tissue with a much lower mitotic rate. Early prophase nuclei have about twice the value of the telophase nuclei, and the peaks coincide with those of the liver classes.

TABLE 4

AVERAGE AMOUNTS OF DNA PER NUCLEUS IN FORMALIN-FIXED TISSUES OF AN ADULT FROG *Rana pipiens*), AS OBTAINED BY PHOTOMETRIC DETERMINATIONS OF FUELGEN PREPARATIONS*

Cell Type	DNA (Feulgen)	Standard Error	No. of Measurements
Spermatids	2.80	.06	20
Lymphocytes	5.60	.06	20
Erythrocytes	6.00	.07	20
Kidney tubule	5.60	.06	20
Small intestine epithelium	5.76	.14	20
Liver Class I	5.60	.09	20
Liver Class II	10.9	.....	3

* Averages are expressed in arbitrary units (see Table 1 and text).

The process, in the pronephros, of DNA synthesis in a dividing tissue is evident from Figure 8. Dividing nuclei already have, at the earliest recognizable prophase, twice the normal Class I amount of DNA. During prophase and metaphase, although these stages are not accurately measurable, the DNA stays constant, since it is divided into two nuclei at anaphase, each with the normal Class I amount. Telophase nuclei are again measurable; these show half the

prophase value. Through much of the interphase the nuclei must keep the Class I amount of DNA, since the majority of the measured resting cells have this value. Then, while still appearing microscopically as a resting nucleus, DNA is built up, and, when the doubled amount is reached, prophase is initiated.

Figure 8, *B*, shows another dividing tissue in *Ambystoma*—the erythrocytes, as measured in the heart region. The distribution of interphase values is roughly similar to the pronephros.

The distribution of values in the embryo mouse liver is essentially similar to that in *Ambystoma*, giving a broad curve (Fig. 9, *D*), with a preponderance of values at the lower end. For comparison,

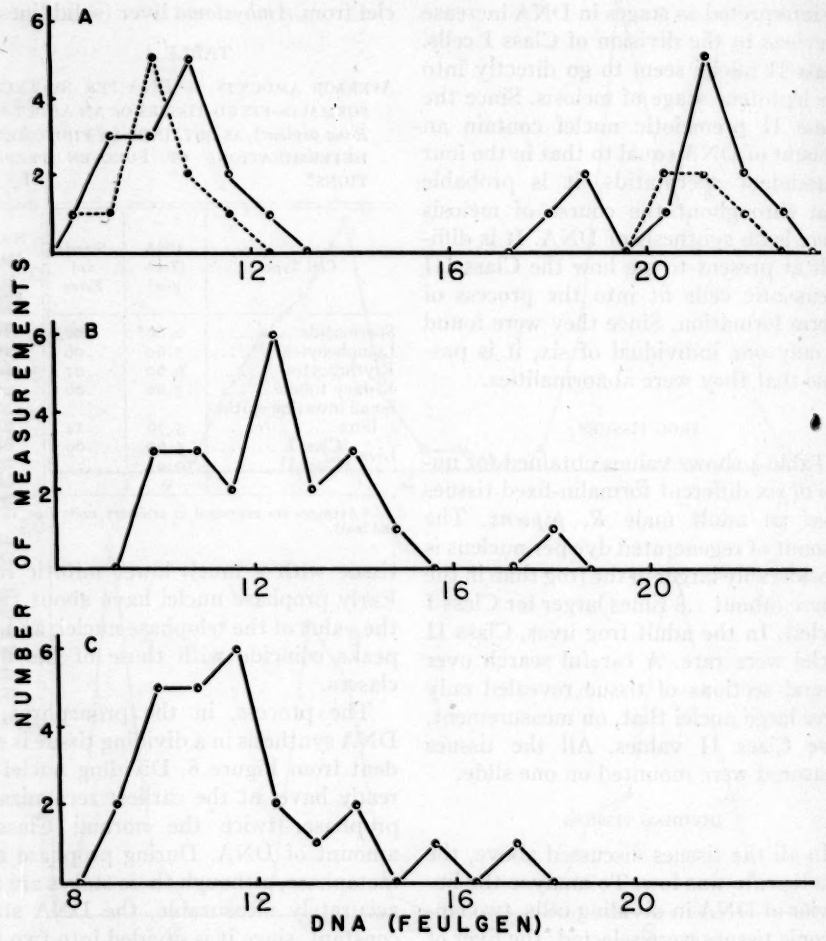

FIG. 8.—Amounts of DNA, in arbitrary units, in individual nuclei of *Ambystoma* larval (hatching stage) tissues. *A*, *solid lines:* liver, showing Classes I and II interphase nuclei; *dotted lines:* telophase (*left*) and prophase (*right*) nuclei from pronephros. *B*, Erythrocyte interphase nuclei from the heart region. *C*, Pronephros interphase nuclei.

two tissue with lower mitotic rates were measured. These were the embryonic erythroblasts (normoblasts), shown in curve *A*, and the Class II lymphocytes, shown in curve *B*. A few intermediate normoblasts were found. Here also it is evident that DNA is built up during interphase before the visible beginning of prophase, since no nuclei have been included in curve *D* that appeared mitotic. Liver telophases are plotted in curve *C* and fall into Class I.

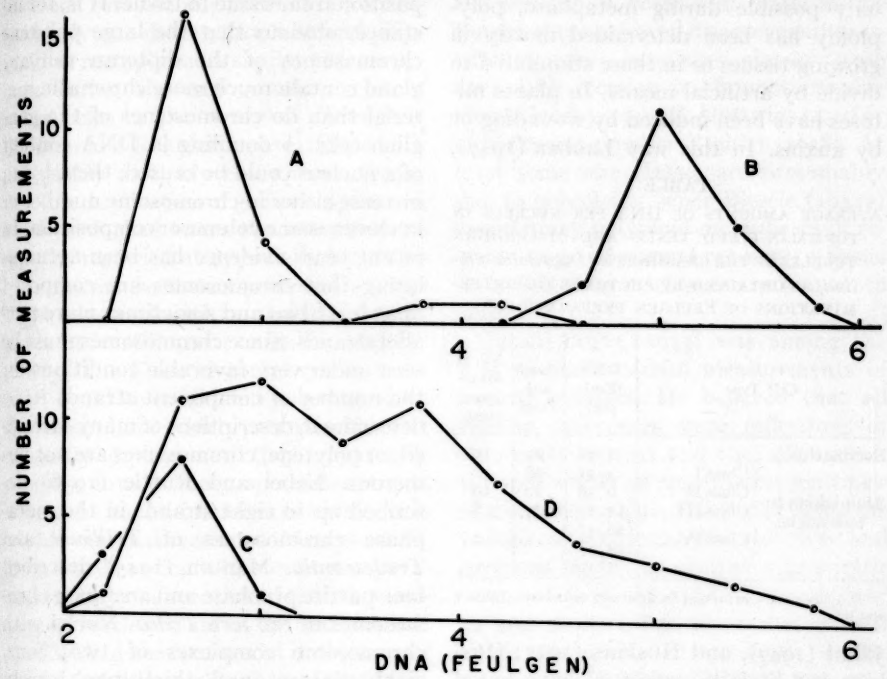

FIG. 9.—The amounts of DNA, in arbitrary units, in individual nuclei of an 11-day mouse embryo. *A*, Erythroblasts from heart and dorsal aorta. *B*, Class II lymphocytes from the same regions. *C*, Telophase nuclei from the liver. *D*, Interphase nuclei from the liver.

GRASSHOPPER TISSUES

The presence of classes of DNA in the Malpighian tubules and adjacent tissue of the grasshopper *Dissosteira carolina* is shown in Table 5. Material was fixed in formalin. Four classes were found; the higher classes were rare, and only a few of these nuclei were measurable.

DISCUSSION

The data presented here support the theory of Boivin, Vendrely, and Vendrely (1948) that the amount of DNA is the same in all the nuclei of an organism. The only exceptions to this rule found in the present study are (*a*) spermatid nuclei contain half the common value; (*b*) some tissues contain nuclei with two, four, or eight times the common amount of DNA; and (*c*) dividing tissues contain nuclei with intermediate values, since a doubling of DNA is associated with mitosis.

It has been known for many years that certain plant and animal tissues con-

tained polyploid nuclei, with two, four, eight, etc., times the normal chromosome number. Nemec in 1904 described polyploidy in root tips, and since that time polyploid nuclei have been described in a variety of tissues, chiefly those of plants. Since chromosome counts are usually only possible during metaphase, polyploidy has been determined mostly in growing tissues or in those stimulated to divide by artificial means. In plants mitoses have been induced by wounding or by auxins. In this way Lauber (1947), Jähnl (1947), and Huskins (1947; Huskins and Steinitz, 1948*a*, *b*) have found polyploidy in differentiated root, leaf, and fruit tissues. Biesele (1944*a*) has described diploid, tetraploid, and octaploid nuclei in regenerating rat liver, determined by chromosomal counts. In a few insect tissues the chromosomes are visible enough in resting cells to make counts possible. Because of this, White (1945) was able to find three different polyploid classes (diploid, tetraploid, and octaploid) in the testis sheath cells of the grasshopper *Schistocerca*. In nuclei of the water strider, *Gerris lateralis*, the heteropycnotic X chromosomes are visible at interphase. By counting these, Geitler (1937, 1939) was able to determine 512-ploid nuclei and possibly higher classes in the giant salivary-gland cells.

TABLE 5

AVERAGE AMOUNTS OF DNA PER NUCLEUS IN FORMALIN-FIXED TESTIS AND MALPIGHIAN TUBULES OF THE GRASSHOPPER *Dissosteira carolina*, AS OBTAINED BY PHOTOMETRIC DETERMINATIONS OF FEULGEN PREPARATIONS*

Cell Type		DNA (Feulgen)	Standard Error	No. of Measurements
Spermatids		1.78	.02	10
Malpighian tubule nuclei	Class I	3.25	.08	10
	Class II	6.36	.22	10
	Class III	14.1	.44	8
	Class IV	26.4 24.7	.....	2

* Averages are expressed in arbitrary units (see Table 1 and text).

Chromosome number, however, need not be a definite indication of the amount of DNA in nuclei, since the chromosomes themselves may vary in volume or composition from tissue to tissue. It is, for instance, obvious that the large polytene chromosomes of the dipteran salivary gland contain much more chromatin material than do chromosomes of the ganglion cells. A doubling in DNA content of a nucleus could be caused, then, by an increase either in chromosome number or in chromosome volume or composition. In recent years evidence has been accumulating that chromosomes are composed of at least two and sometimes more parallel strands. Since chromosomes must be seen under very favorable conditions for the number of component strands to be determined, descriptions of many-stranded, or polytene, chromosomes are not numerous. Nebel and Ruttle (1936) described up to eight strands in the metaphase chromosomes of *Trillium* and *Tradescantia*. Manton (1945) described four-partite prophase and anaphase chromosomes in the fern *Todea*. Nuclei with chromosome complexes of two, four, eight, sixteen, and thirty-two loosely united strands have been described by Berger (1938) and Grell (1946) in the larval ileum of the mosquito *Culex pipiens*. Other desciptions of polyteny in chromosomes are summarized by Kaufmann (1948). Biesele (1945) and Biesele and Poyner (1943) believed that the degree of polyteny is higher in certain neoplastic than in normal tissues. They measured chromosome size in cancer tissues of rabbit and man and found chromosome volume to fall into classes one, two, and four times larger than those of normal cells.

In most resting nuclei, determination

either of polyploidy or of polyteny is impossible. Indeed, the distinction between the two types of chromatin duplication, when applied to interphase nuclei, seems somewhat arbitrary. Berger (1938) described the falling-apart of polytene-like complexes in *Culex* preceding metaphase, in which case the nuclei could be considered polytene during prophase and polyploid at metaphase. A similar process occurs during the endomitotic cycles of nurse cells in the ovary of *Drosophila* (Painter and Reindorp, 1939). Since both polyploidy and polyteny apparently result in a duplication of genic components, in physiological terms the two processes may be regarded as essentially identical.

In the mouse tissues measured here the DNA classes found in the liver would seem, at least in part, to be due to polyploidy. The Class II gonial nuclei, however, could be considered polytene, since the metaphase which precedes them is normally diploid and they apparently progress into primary spermatocytes in which the haploid number of bivalents is visible at diakinesis or Metaphase I. From studies of corn tissues now in progress it might be mentioned that the commonest nuclear class in the leaf has half the DNA of the root nuclei in the zone of elongation. Since metaphases in both tissues are diploid, the root chromosomes would seem to have a higher degree of polyteny. It should be pointed out, however, that an increase in amount of DNA, not correlated with an increase in chromosome number, need not be an indication of polyteny. Polyteny is, by definition, a morphological concept. It is conceivable that chromosomes could exist with different amounts of DNA and yet, as far as shown by visible evidence, contain the same number of component strands. In plant tissues Huskins (1947) has reported finding, on morphological grounds, "every type of intermediary and combination of polysomaty and polyteny."

Many authors have found that the nuclei of certain tissues fall into classes according to volume. It has been assumed by several workers that there was a relation between nuclear volume and the amount of chromatin material, although there has been disagreement as to whether the large-size classes were caused by increase in chromosome size alone (Jacobj, 1935; G. Hertwig, 1939) or chromosome number (Geitler, 1938). At least some size classes are presumably due to polyploidy, since Biesele (1944*a*) found many polyploid metaphases in regenerating and normal rat liver, a tissue in which volume classes are known to occur.

Jacobj (1925, 1935) was among the first to make careful measurements of nuclear volumes. He believed that all volume differences were reflections of chromatin content and that all nuclear volumes within an animal were multiples of a common value. Taking as unity the volume of a common class of liver and pancreas nuclei, he erected a geometric series of $\frac{1}{8}$, $\frac{1}{4}$, $\frac{1}{2}$, 1, 2, 4, 8, 16, and 32 and showed that the nuclei of each tissue approximated one value in the series. This relationship was not confirmed for rat tissues by Biesele (1944*b*), who found intermediate nuclear volumes. Intermediates were attributed by G. Hertwig (1938) to certain physiological factors acting on nuclear size. In five tissues Jacobj found more than one size class. These were liver, pancreas, thymus lymphocytes, testis, and spinal ganglion. The occurrence of volume classes has been confirmed, particularly for the liver, by several workers, including Sulkin (1943), Biesele (1944*b*), and Schreiber (1946).

In the present study, classes of amounts of DNA have been found in

liver, pancreas, sex, and Sertoli cells of the testis and in thymus and blood lymphocytes. Spinal ganglion cells were not measured. Within any one of these tissues the relationship between amount of chromatin and nuclear volume has been confirmed, although volume classes may overlap rather widely where DNA classes do not. In Figure 6, *A*, are plotted the volume distribution of the same 85 adult liver nuclei shown in Figure 6, *B*. The means of the volumes fall into the same 1:2:4 ratio shown by the amounts of DNA, but the classes are less sharply defined.

When nuclei of different tissue types are compared, however, there is no correlation between volume and DNA content. In the present work the smallest nuclei measured (normoblasts) and the largest (motor neurones) contained the same amount of DNA, although the volume of one was about twenty times the other. Larger neurone nuclei were not measured, since they were cut in sectioning, but by inspection it appeared likely that they were also diploid.

Schreiber (1946) measured nuclear volumes in the testes of several snake species, showing that primary spermatocytes, secondary spermatocytes, and spermatids occur in a volume ratio of 4:2:1, respectively, and that two classes of gonia occur with a ratio, in the same units, of 2 to 3. He, therefore, assumed that during the formation of primary spermatocytes the gonia are first diploid and then pause in a "sesquiphase," in which half the chromosomes are polytene. As demonstrated in the present paper, spermatogonia and spermatocytes of the mouse testis show approximately the volume relations described by Schreiber for the snake. The Class II premeiotic nuclei, however, have the same DNA content as do the larger primary spermatocytes (Fig. 7), so that there is no evidence for a class of nuclei with an intermediate chromatin content. From the present study it is evident that, except within the confines of one cell type, nuclear volume is not necessarily an indication of chromatin content.

O. Hertwig in 1918 was probably the first to state in unequivocal terms the theory that the chromosomal content of all nuclei in an organism is identical. Although this concept has been widely accepted, it has been, nevertheless, tempting to imagine that some sort of somatic gene segregation occurred during development, as a guide to differentiation. This idea has been recently re-examined by Huskins (1947) and Huskins and Steinitz (1948*a*, *b*), who have pointed out the numerous known exceptions to Hertwig's concept of chromosomal stability. Most of these exceptions involve either polyploidy or polyteny, and thus Huskins has considered the possible role of quantitative gene differences as a factor in differentiation.

At least in the animal tissues studied here, the occurrence of DNA classes higher than the normal diploid amount, presumably caused by polyploidy or polyteny, does not seem a vital factor in differentiation. In all tissues in which Classes II and III nuclei were found, Class I nuclei were also present. Also, in the case of mouse liver, the Class I nuclei are commonest in young, but already differentiated, tissue, and most of the higher classes appear long after differentiation has occurred. Whether or not polyploidy or polyteny is associated with differentiation in other forms, such as insects, has yet to be determined.

As mentioned above, the analyses of mammalian nuclear suspensions made by Mirsky and Ris (1949), when interpreted as the average amount of DNA per nucleus, failed to show a simple and constant relationship. The amount of DNA

per nucleus, instead of being exactly twice that found in sperm, was found to vary from two and two-tenths times in calf liver to three times the sperm value in beef liver. This led Mirsky and Ris to the belief that mammalian tissues may involve a special type of partial duplication of gene material (not found in other vertebrates, where the DNA ratios between somatic nuclei and sperm were more nearly 2 to 1). If a certain number of nuclei in the mammalian suspensions studied belonged to Class II or Class III, then the average amount per nucleus would be raised. The authors have stated that the data which they present were corrected for "the double-size nuclei," and these are, presumably, nuclei of Class II. Work on beef tissues now in progress by the author, using the techniques here described, has failed to show intermediate values of the magnitude indicated by Mirsky and Ris. In fact, the photometric data from beef nuclei, though preliminary, are in complete agreement with the results obtained for mouse tissues, the only important difference being that the DNA classes, in liver at least, occur in somewhat different frequencies. In the beef liver studied, Class II nuclei were not common, and Class III nuclei were rare or absent. Beef spermatid nuclei have been found to contain approximately half the Feulgen dye per nucleus occurring in somatic tissues. These measurements are in disagreement with the gross chemical determinations made by Mirsky and Ris on ripe spermatozoa and are in better agreement with the determinations of Vendrely and Vendrely (1948). At present, photometric data lend no support to the theory, expressed by Mirsky and Ris (1949) and reiterated by Mirsky (Ris and Mirsky, 1949*b*), that mammalian tissues involve a partial genic duplication not found in other vertebrates.

Schrader and Leuchtenberger (1949), in a photometric study of Feulgen-stained *Tradescantia* tissues, also failed to find well-developed classes of DNA and have thus reached the conclusion that "the amount of DNA carried in a given chromosome may vary." For example, the relative values obtained for root tip, leaf, and bud tapetum interphase nuclei are, respectively, 5.5, 9.0, and 12.0. Work on plant tissues now in progress by the author is not in agreement with Schrader and Leuchtenberger. Several hundred corn nuclei have been measured, and intermediate values of the type reported by these workers have not been found. In fact, the material so far measured agrees well with the animal data in showing constant amounts of DNA per nucleus within the tissues of one plant. For example, the microgamete nuclei of the mature corn pollen grain average half the DNA in the smallest somatic nuclei, and well-defined classes with two, four, eight, sixteen, and thirty-two times the haploid value have been found.

Although several authors have presented theories as to the time of gene doubling in cell division, very few quantitative data have been published. During spermatogenesis in the grasshopper *Gomphocerus*, Caspersson (1939) found that the DNA per nucleus was approximately constant from late leptotene to the latest stage studied, which was diplotene. Ris (1947), on the other hand, described a gradual increase in DNA during the meiotic prophase of the grasshopper *Chortophaga*, so that the metaphase chromosomes had almost twice the DNA found in leptotene. Ris described a similar increase during the mitotic prophase in onion root meristem. Both these authors used photometric determinations; Caspersson studied living spermatocytes with ultraviolet light, and Ris

used ultraviolet light and Feulgen dye on fixed preparations. The data presented by Ris are subject to certain errors, since very few nuclei were measured and, owing to the technique, there were probably large distributional errors caused by the contracted chromatin of late prophase, metaphase, and anaphase.

In the dividing tissues studied in the present work (mouse liver and testis and salamander pronephros) DNA had already doubled in amount before the onset of the meiotic or mitotic prophase. In studies on the DNA in plant tissue, however, to be published shortly, DNA increase in the corn root meristem takes place in the resting nuclei shortly after the conclusion of telophase. The situation in the corn root thus differs slightly from that found in the animal tissue studied, since in one case DNA increases before prophase to twice the normal amount and in the other case DNA is reduced to half the normal amount by division and is built up after telophase. Both processes are alike in one respect, namely, that DNA synthesis does not occur during the mitotic stages. Mitoses in the corn root are possibly complicated by the fact that most resting nuclei fall into Class II. In the leaf meristem, where Class I nuclei are dividing, the situation seems more nearly like that in the animal tissues studied. Seshachar (personal communication) has recently found that the micronucleus of the ciliate protozoan, *Chilodonella uncinatus*, soon after mitosis shows a value indicating a halving of the normal; the situation thus seems similar to that found in the corn root.

The present study, as well as those of Boivin, Vendrely, and Vendrely (1948), Vendrely and Vendrely (1948, 1949), Mirsky and Ris (1949), and Ris and Mirsky (1949*b*), shows that DNA is maintained in constant amounts within the cell. A more accurate picture of exactly how much the DNA varies between nuclei cannot be determined with present equipment. In respect to photometric determinations, it should be stressed that the variability shown in the measurements obtained is approximately equal to the error expected from the operational variables discussed above. There is no indication that, in differentiated, nondividing tissues, the scattering of measured values is caused by a natural variability of the amount of DNA in nuclei. In fact, the more nearly ideal are the conditions of measurement, the lower is the standard deviation of the values obtained.

In agreement with the quantitative stability shown by DNA is its remarkable chemical stability, as shown by studies with labeled phosphorus and nitrogen. Brues, Tracy, and Cohn (1946), Brown (1948), and Bergstrand *et al.* (1948) have shown that DNA has an exceedingly low rate of turnover in nondividing tissues, many times slower than that of the ribose nucleic acid fraction of the same material.

The chemical and quantitative stability of DNA in somatic nuclei and its reduction during spermatogenesis lend support to the theory, as expressed by Boivin *et al.* (1948), that "chaque gène aurait pour constituant essentiel une macromolécule d'un acide désoxyribonucléique particulier." Whether DNA has all the properties which we expect genes to possess has yet to be determined. For the present, its quantitative behavior in animal tissues seems best interpreted by the view that DNA is an essential gene component.[5]

[5] After preparation of the present manuscript, papers by Lison and Pasteels (1949) and Pasteels and Lison (1950) have come to my attention. These authors have made photometric determinations on Feulgen-stained nuclei of mole testes and of several rat tissues, with results that differ somewhat from those presented here. Although measurements of

SUMMARY

1. Methods of absorption spectrophotometry as applied to individual Feulgen-stained nuclei have been redescribed, with a discussion of the accuracy and major variables of the technique. Limits of accuracy were usually determined by the type of nuclei measured. Where absorbing material was uniform in distribution and nuclear volumes were readily determinable, standard deviations of approximately 5 per cent have been obtained for a series of measurements.

2. The use of the Feulgen reaction is discussed for making quantitative estimates of the desoxyribose nucleic acid in animal nuclei. Although the Feulgen reaction, if properly used, is specific for DNA, a number of variables influence the dye intensity in cytological preparations. In the present work these variables were found difficult to control, so that variations up to 15 per cent between preparations of similar material were occasionally obtained, even with carefully controlled conditions. It was concluded, however, that the Feulgen reaction gave an accurate relative measure of the DNA in the nuclei of any one preparation. Extinctions of Feulgen-stained nuclei were shown to be in direct proportion to thickness (Lambert's law), and for any one cell type the relation between nuclear volume and extinction was precisely that expected on the assumption that DNA occurs in a constant amount per nucleus and that changes in concentration of DNA are accurately reflected (Beer's law). Thus the method can give accurate relative estimates, although the Feulgen reaction cannot by itself provide data on the absolute amounts of DNA in nuclei. For this reason, values in the present study are presented in convenient arbitrary units.

3. Nuclei were measured in ten different formalin-fixed somatic tissues from young and adult mice. All nuclei, regardless of tissue type, showed approximately the same (Class I) amount of DNA, except for some of the nuclei in liver, pancreas, thymus, blood lymphocytes, and Sertoli cells, which contained two (Class II) or four (Class III) times the common amount. Occasional rare intermediate values were found, presumably associated with mitoses. In liver of a 10-day-old mouse, Class I nuclei occurred in the greatest numbers; in the adult mouse liver, Class II nuclei were somewhat more abundant.

4. Mouse spermatid nuclei contained half the DNA found in Class I somatic nuclei. Secondary spermatocytes contained twice, and primary spermatocytes contained four times, the spermatid value. Each primary spermatocyte was thus found to contain all the DNA necessary for the production of four spermatids. Nuclei of premeiotic sex cells were found to be of two types, a smaller class containing the diploid Class I amount of DNA and a larger (Class II) type, containing twice the amount. Class I premeiotic cells were most common in testes of mice 1–10 days old. With maturity, Class I nuclei became less frequent and

primary spermatocytes and spermatids gave the 4:1 ratio reported by Swift (1949), values for somatic tissues varied from the expected diploid amount to values about 30 per cent below this in adipose nuclei and in the smallest nuclei of liver and pancreas. Within the pancreas and liver, however, well-marked nuclear classes were obtained. To explain these discrepancies from the theory of Boivin *et al.* (1948) Pasteels and Lison have suggested that chromatin diminution must occur in some somatic tissues (an opinion, incidentally, exactly opposite to that of Mirsky and Ris [1949], who postulated partial DNA duplication in beef tissues to account for values that were apparently too high). Unpublished data on testes, liver, and pancreas of white rats (Wistar and Sprague-Dawley strains) obtained by the present author do not show the discrepancies reported by Pasteels and Lison. A description of the cytological and photometric techniques used by these authors has not yet been published.

Class II nuclei commoner; the Class II nuclei apparently proceed directly into leptotene of the first maturation division.

5. The nuclei of six formalin-fixed frog tissues were measured from an adult male *R. pipiens*. All somatic nuclei measured contained approximately the same amount of DNA (slightly less than twice the amount found in mouse nuclei) except for a few liver nuclei, which contained twice the common amount. The spermatids contained half the DNA found in most somatic nuclei.

6. The behavior of DNA during mitosis was studied in developing liver tissue of an 11-day mouse embryo and in pronephros and erythrocyte nuclei of a recently hatched *Ambystoma* larva. Interphase nuclei of dividing tissues showed a large number of DNA values intermediate between Class I and Class II amounts. All early prophase nuclei measured fell into the Class II group, whereas all telophases fell into Class I. It thus appears, in the tissues studied, that DNA for cell division builds up in the interphase nucleus before the visible onset of prophase. During the visible stages of mitosis, no DNA is synthesized, and the amount is cut in half at anaphase.

7. Four classes of DNA were found in the Malpighian tubule nuclei of the grasshopper *Dissosteira carolina*, with the ratio 1:2:4:8. Spermatid nuclei contained half the amount of DNA found in the smallest class.

8. No correlation was found between the amount of DNA in a nucleus and the nuclear volume, except within the limits of one particular tissue. The mouse nuclei falling in Class I covered a volume range of about twenty times, from the smallest to the largest measured.

9. The quantitative behavior of DNA in a variety of animal species, as indicated by photometric determinations of Feulgen-stained nuclei, lends support to the concept that DNA is an essential component of the gene.

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All photographs are of Feulgen-stained material. All tissues pictured were fixed in 50 per cent formalin except that shown in Plate II*D*, which was Carnoy-fixed.

PLATE I

FIG. A.—Newborn mouse testis, showing tubule containing Class I premeiotic nuclei.

FIG. B.—Newborn mouse testis, showing tubule with spermatogonial prophase (*P*) and metaphase (*M*), and Classes I and II premeiotic nuclei.

FIG. C.—Newborn mouse testis. Tubule in longitudinal section, showing Classes I and II premeiotic nuclei.

FIG. D.—Sixteen-day mouse testis, showing Class II premeiotic nuclei with fibrous appearance, merging into leptotene stage.

FIG. E.—Sixteen-day testis, showing tubules with Classes I, II, and III premeiotic nuclei.

FIG. F.—Detail of upper central part of *E*, showing Class III nuclei.

PLATE II

FIG. A.—Adult mouse testis, showing the typical way in which Class II premeiotic cells line the tubules.

FIG. B.—Portions of two adult mouse tubules, one showing a few Class I premeiotic nuclei.

FIG. C.—Pancreas of an adult mouse, showing the nuclear classes.

FIG. D.—Pronephros of a larval (hatching stage) *Ambystoma*, showing the high frequency of mitotic figures.

PLATE I

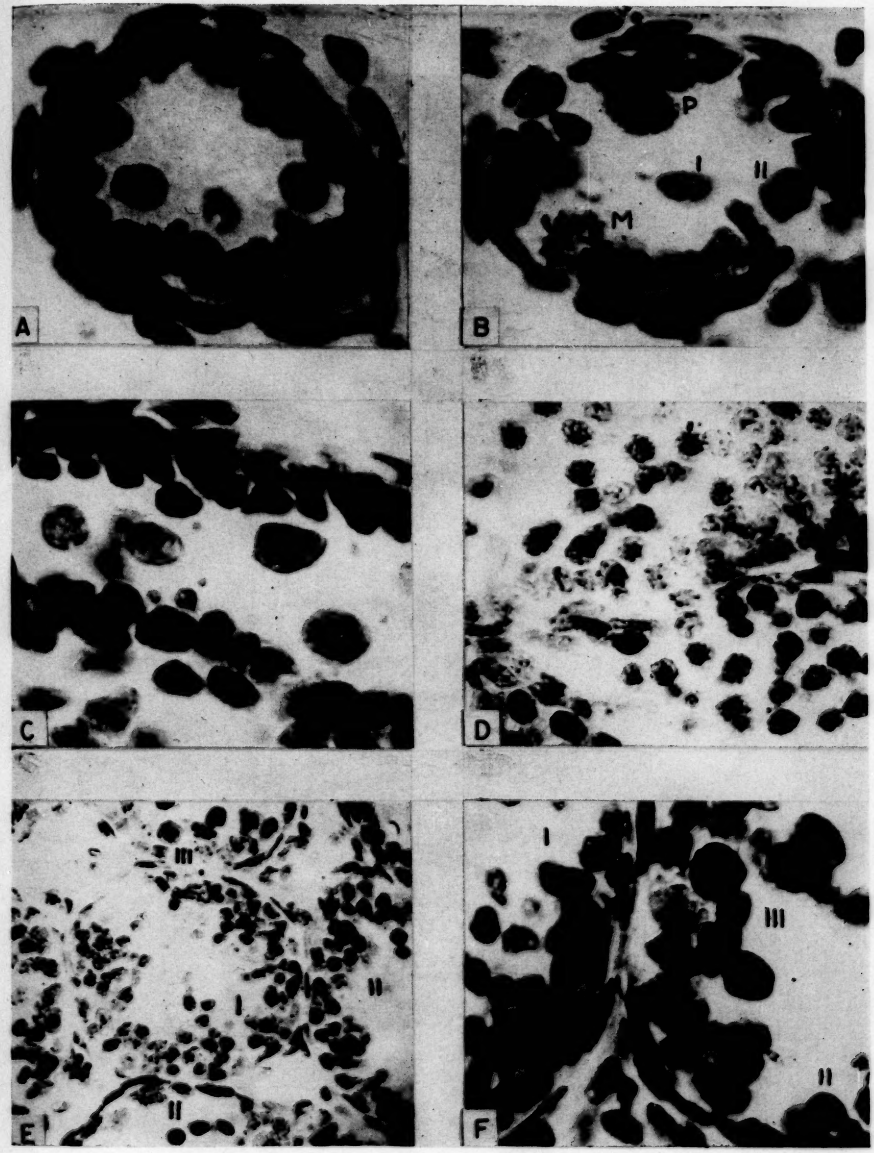
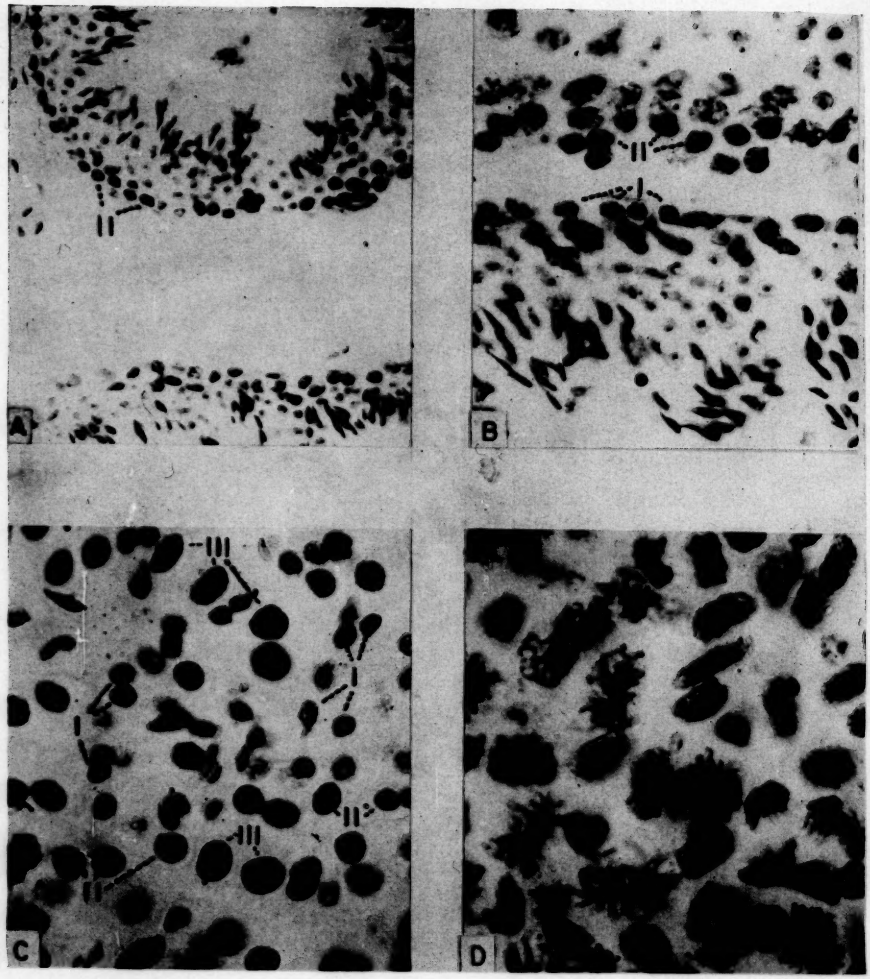


PLATE II



THE RESISTANCE OF CELL MEMBRANES TO INTERNAL PRESSURE[1]

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ALTHOUGH most living cells are in osmotic equilibrium with the surrounding medium, it is possible for a considerable difference between the internal and external osmotic pressure to exist. For example, cells may be placed in dilute solutions whose osmotic pressure is considerably lower than that of the cell interior. When unfertilized eggs of the sea urchin, *Arbacia punctulata*, are placed in dilute sea water, they will swell until they reach osmotic equilibrium. Whereas plant cells are able to resist expansion by virtue of their rigid cellulose walls, animal cells during osmotic swelling have no containing walls to protect them.

A common belief exists among many biologists that the delicate membranes of living cells may resist very high pressures, that is to say, pressures of the order of magnitude of several atmospheres. Even though in some cases there may be an osmotic-pressure difference of several atmospheres between the cell interior and the external medium, this does not necessarily mean that the plasma membrane itself is exposed to such pressures. In order to investigate this problem, the present study was undertaken in an attempt to determine the maximum pressure which cell membranes are able to withstand from within.

This problem was suggested by Dr. L. V. Heilbrunn, and I wish to express my appreciation for his advice and criticism.

MATERIAL AND METHODS

The experiments were performed on the unfertilized eggs of the sea urchin, *A. punctulata*, the starfish, *Asterias vulgaris*, and the worm *Chaetopterus pergamentaceous*. Starfish eggs were obtained by removing the ovaries from ripe females and placing them in finger bowls of sea water. The loose eggs were then strained through a double layer of cheesecloth in order to remove all debris and were subsequently washed three times in sea water. Sea-urchin eggs were obtained from gravid females whose tests had been transected in order to stimulate the shedding of eggs into Stender dishes filled with sea water. The jelly layer investing the eggs was removed by gently moving a 20-ml. suspension of the eggs from side to side five times in a 100-ml. cylinder. The eggs were then washed four times in sea water. The eggs of *Chaetopterus* were obtained by removing several parapodia from a female and cutting them in a Stender dish filled with sea water. They were then passed twice through a double layer of cheesecloth in order to remove the mucus and debris and were washed

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three times in sea water. The freshly prepared egg suspensions were placed in Stender dishes and stored in a constant-temperature water bath. The temperature of the bath was 21° ± 0.1° C. The sea water used in the experiments was filtered. Unless otherwise noted, all the eggs used in any one set of experiments were those of a single female.

A method was devised for measuring the amount of pressure necessary to rupture the cell membrane when eggs were micro-injected with sea water. The method for micro-injection and measurement of pressure was similar to that of Kopac for nonaqueous solutions (1943). The apparatus consisted of a Chambers micromanipulator and micro-injection apparatus, with a diaphragm-type pressure gauge attached in close proximity to the micropipette holder. A delicate micrometer screw attachment activated the plunger of the micro-injection syringe in order to control the pressure very accurately. Uniformity in form and size of the micropipettes was achieved by producing them with a Livingston automatic needle-pulling machine (Chambers and Kopac, 1937). The tip of the micropipette was broken against a cover slip on the roof of the micromanipulating moist chamber. The orifice thus produced rarely exceeded 2μ in diameter. Eggs were prepared in small hanging drops on a second cover slip and brought into the field of the microscope. The micropipette was inserted into the cell, and the micrometer screw activated by hand. At each half-revolution the turning of the screw was stopped for an instant in order to build up the pressure gradually. In order to ascertain exactly the pressure at the instant at which the cell membrane burst, it was necessary to be able to see the face of the gauge dial at the same time as the egg. This was made possible by projecting the face of the gauge dial into the field of the microscope by means of a camera lucida. As soon as the cell membrane ruptured, the pressure indicated on the dial was read and recorded. The micropipette was then rinsed with injection fluid, refilled, and, if found to be still functional, used on another cell. Only when the orifice of the pipette was in any way damaged or obstructed by debris was a new micropipette inserted. In general, one micropipette was useful for approximately three or four determinations. No more than about a half-dozen eggs were tried in any one hanging-drop preparation before a new preparation was made from the egg suspension on the water bath. The injection fluid, in all cases except in the experiments where eggs were exposed to dilute sea water, was freshly filtered sea water. In the latter experiments the diluted sea water was micro-injected. After obtaining a large number of control values on untreated eggs, i.e., eggs in normal sea water, various series of determinations were made on eggs exposed to different experimental solutions or treatments. All the experimental solutions were prepared in fresh, filtered sea water. The pH of egg suspensions in normal sea water and in experimental media was taken after the experiments had been performed. Measurements of pH were made with a Beckman glass-electrode pH meter. This was done in order to ascertain whether the results of any of the experimental treatments were due to the treatments themselves or to changes in pH.

Before the actual experiments were begun, numerous preliminary determinations were made in order to make certain that repeatable results could be obtained. In general, it was possible to obtain repeatable results for each set of ten determinations under any one set of experimental conditions.

In the series of experiments where isosmotic solutions of cations were studied, the final three washings of the eggs were made in the respective experimental solutions. The alkaline $CaCl_2$ solution was obtained by adding borate buffer whose concentration was 0.027 m. The egg suspensions in the experimental solutions were then stored in the water bath before tests were begun.

The lamp used for exposure of eggs to ultraviolet radiation was of the Westinghouse type, delivering 90 per cent of its radiation in the 2730 Å band. The distance between the lamp and the eggs was approximately 23 cm.

In exposing eggs to various concentrations of anesthetics, the customary washings were made in sea water. After the final washing the eggs were allowed to settle to the bottom of the Stender dish, and 5 drops of concentrated egg suspension thus obtained were added to 20 ml. of anesthetic containing sea water. The suspension was then placed in the water bath.

Various forms of the proteolytic enzyme trypsin were tried. The crude trypsin was obtained from the Fisher Scientific Company. Partial purification was achieved by centrifuging the trypsin containing sea water. The pure trypsin was a crystalline preparation obtained from the Armour Company. Inactivated solutions of trypsin were prepared by heating the trypsin solutions at 80° C. for 30 minutes over a water bath. The method of exposure of the eggs was to add concentrated egg suspension to an excess of the enzyme solution in the constant-temperature water bath.

The effect of hypotonic sea water was studied in the following manner. The eggs were washed four times in normal sea water as usual, and the egg suspensions were stored in the water bath. Dilute sea-water solutions of varying concentrations were prepared by adding distilled water in varying amounts to sea water in order to obtain the desired percentage dilutions. The hypotonic solutions were then placed in the water bath at constant temperature, and concentrated egg suspension was added to an excess of hypotonic medium. In all cases the eggs were exposed to the hypotonic media for 30 minutes before experiments were begun, in order to assure the attainment of equilibrium volume. The swelling to equilibrium volume was followed by measuring the diameter of the eggs with the aid of a calibrated ocular micrometer. The resistance of the membrane to internal pressure was then determined, using the hypotonic sea-water solutions as injection media.

The preparation of sea water of varying pH was accomplished as follows: Twenty-four ml. of 0.1 N HCl was added to sea water to make a total of 1,000 ml. This solution was placed in large, shallow bowls and allowed to stand exposed to the atmosphere for *ca.* 27 hours at room temperature in order to remove all carbon dioxide present in the sea water. To each 10 ml. of this modified sea water varying numbers of drops of 0.1 N HCl and 0.1 N NaOH were added in order to obtain a range of solutions of varying pH. On the following day, eggs washed and concentrated in the usual way were exposed to an excess of the above solutions in the water bath. The eggs were then tested, and the pH of each of the solutions was determined immediately before and after the tests performed in each solution.

EXPERIMENTAL RESULTS

Table 1 summarizes the results of internal-pressure resistance determinations for the membranes of three different species of marine eggs. In each case, eggs from two females of a given species were

studied. In all cases and in all the subsequent studies, the egg surface, immediately upon breaking, was found to seal itself with a typical self-sealing reaction, i.e., a surface-precipitation reaction. In the case of the *Chaetopterus* egg, the egg membrane was found to rupture twice.

TABLE 1

RESISTANCE OF UNFERTILIZED EGGS IN SEA WATER TO INTERNAL PRESSURE

Kind of Egg	No. of Determinations	Pressure (Mm. Hg.)
Arbacia	36	11.9±4.5
Asterias	40	8.8±5.6
Chaetopterus		
First rupture	40	6.6±2.8
Second rupture	20	24.2±3.1

TABLE 2

EFFECT OF ISOSMOTIC SOLUTIONS OF CATIONS ON RESISTANCE OF *Arbacia* EGGS TO INTERNAL PRESSURE

Solution	pH	Pressure (Mm. Hg.)
0.53 M NaCl	6.0	3.8± 2.3
0.53 M KCl	9.2	3.0± 2.2
0.30 M $CaCl_2$	5.9	25.3±10.2
0.30 M $CaCl_2$	8.2	39.2± 9.9
0.30 M $MgCl_2$	6.0	21.8±12.8
M/27,000 $AlCl_3$*	6.7	25.6±10.2

* In Ca-free sea water.

The first rupture occurred at the site of polar-body formation. When the pressure of the micro-injection fluid was not stopped after the first rupture, the egg was found to be able to withstand a further increase in internal pressure until a second rupture, not necessarily at the site of the first rupture, occurred.

The results of the effect of isosmotic solutions of cations are shown in Table 2. The average of pressure determinations of twenty eggs for each solution is shown. Of the common cations present in sea water, the bivalent cations, calcium and magnesium, increased the resistance of *Arbacia*-egg membranes to internal pressure. Alkalinity increased the effectiveness of calcium. By far the most potent cation in this respect, the trivalent aluminum ion, had to be used in very dilute concentration. Solutions of aluminum chloride of a concentration greater than M/27,000 were difficult to work with. In such solutions the eggs became sticky and adhered to the glass. The univalent cations, sodium and potassium, decreased the resistance of the membrane.

TABLE 3

EFFECT OF EXPOSURE TO ULTRAVIOLET LIGHT (2730 Å) ON RESISTANCE OF *Arbacia* EGGS TO INTERNAL PRESSURE

Time of Exposure (Minutes)	Pressure (Mm. Hg.)
1	4.6±2.3
2	5.4±2.7

When eggs were exposed to ultraviolet light (2730 Å), the egg membranes became weakened, and the internal-pressure resistance showed a considerable decrease. Exposures of longer duration than 2 minutes had no further effect. The results in Table 3 were averages of twenty determinations for each exposure time.

Some anesthetic solutions increased the resistance of the *Arbacia*-egg membrane to internal pressure. Others had the opposite effect. Not much change was noted in the membrane, using 1 per cent ethyl urethane. When its concentration was doubled, however, the membrane became more resistant. Ether, *n*-butyl alcohol, and potassium cyanide all weakened the membrane. An average of twenty determinations for eggs in each of the reagents is shown in Table 4.

Trypsin (Table 5) definitely rendered the *Arbacia*-egg membrane less resistant to internal pressure. That the effect was attributable to the enzymatic activity of the trypsin itself is indicated by the fact that the pressure values were lower with both the crude- and the crystalline-trypsin solutions and that the normal pressure resistance was restored by heat inactivation of the enzyme. The table shows an average of twenty determinations in each solution.

TABLE 4

EFFECT OF ANESTHETICS ON RESISTANCE OF *Arbacia* EGGS TO INTERNAL PRESSURE

Solution	pH	Pressure (Mm Hg.)
1 per cent ethyl urethane..	8.3	12.9±5.0
2 per cent ethyl urethane..	8.2	22.9±6.7
per cent ether.....	8.2	4.8±1.9
1 per cent *n*-butyl alcohol..	8.2	7.0±3.2
2 per cent *n*-butyl alcohol..	8.2	3.2±0.9
0.005 per cent KCN.....	8.1	7.2±2.5

TABLE 5

EFFECT OF TRYPSIN ON RESISTANCE OF *Arbacia* EGGS TO INTERNAL PRESSURE

Solution (0.3 Per Cent)	pH	Pressure (Mm. Hg.)
Crude trypsin.....	6.7	6.4±2.0
Crude trypsin, partially purified.....	6.9	6.6±1.1
Crude trypsin, heat inactivated.....	6.6	13.3±4.7
Crystalline trypsin.....	7.2	5.4±3.1
Crystalline trypsin, heat inactivated.....	7.0	11.5±4.5

In Table 6 are summarized the pressure-resistance determinations on *Arbacia* eggs at equilibrium volumes in sea-water dilutions of varying tonicities. In 90 per cent sea water (90 parts sea water plus 10 parts distilled water) the resistance rises, then reaches a peak in 80 per cent sea water, after which it drops sharply. Eggs from two females were used. A similar series of experiments was carried out on *Chaetopterus* eggs, and the results are presented in Table 7. The temperature of the water bath was 23° C. All the eggs used were those of a single female. The pressure value for each dilution of sea water represents an average of ten determinations. Pressure readings for the first rupture (site of polar-body formation) were taken. The results are similar to those on *Arbacia* eggs in Table 6, except that the pressure value does not drop below the control value until the 60 per cent dilution of sea water.

TABLE 6

EFFECT OF HYPOTONIC SEA WATER ON RESISTANCE OF *Arbacia* EGGS TO INTERNAL PRESSURE

Concentration of Sea Water (Per Cent)	Average Equilibrium Diameter (μ)	Number of Eggs Tested	Pressure (Mm. Hg.)
90.....	76.2	15	15.9±3.3
80.....	79.6	15	21.8±3.2
70.....	81.6	15	6.3±1.9
60.....	85.6	15	2.9±1.1
50.....	89.8	10	2.1±0.4

TABLE 7

EFFECT OF HYPOTONIC SEA WATER ON RESISTANCE OF *Chaetopterus* EGGS TO INTERNAL PRESSURE

Concentration of Sea Water (Per Cent)	Average Equilibrium Diameter (μ)	Pressure (Mm. Hg.)
90.....	104.8	7.5±1.9
80.....	107.4	12.1±3.3
70.....	110.0	17.8±4.0
60.....	116.2	6.4±2.1
50.....	124.2	4.7±1.5

The pH effect on the resistance of the *Arbacia*-egg membrane is presented in Table 8. On the extreme acid side of neutrality, the pressure resistance is very high. Between pH 6.3 and neutrality the resistance drops below the control value

in normal sea water (pH 8.1–8.2 at Woods Hole).

DISCUSSION

There are occasional references in the literature which deal with the resistance of the cell surface to mechanical stresses. For example, Goldforb and Schechter (1932) measured the resistance of the membrane of aging *Arbacia* eggs by noting the time it took for the eggs to burst in 30 per cent sea water. Harvey (1931) determined the tension at the surface of marine eggs from the centrifugal forces necessary to pull the eggs apart. Cole (1932) compressed *Arbacia* eggs with a small gold beam, and from the force of compression and the resulting form of the eggs he determined the tension at the surface of the eggs. Even though the osmotic-pressure relations of marine eggs have been extensively studied (see, e.g., the review article of Lucké and McCutcheon, 1940), no direct method of measuring the resistance of cell membranes to internal pressure has been available.

TABLE 8

EFFECT OF pH ON RESISTANCE OF *Arbacia* EGGS TO INTERNAL PRESSURE

pH	Pressure (Mm. Hg.)
2.4	39.4 ± 15.4
3.2	24.8 ± 5.9
4.2	21.2 ± 4.7
5.9	17.7 ± 5.2
6.3	17.5 ± 4.2
7.0	10.9 ± 2.2
7.7	10.1 ± 3.2
8.4	7.5 ± 2.4
9.6	7.2 ± 4.1

In interpreting the results of the present investigation, some consideration must be given to the method employed. During the course of preliminary experiments, it was feared that the pressures recorded on the gauge were higher than the pressures acting within the cell, for the flow of fluid through the micropipette would tend to reduce the pressure existing at its orifice. This difficulty was resolved by applying the pressure very slowly and gradually. Furthermore, the volume of fluid injected into the cell was relatively small. These two considerations provided for the existence of true hydrostatic conditions. Only if such true hydrostatic conditions are maintained will the pressure at the orifice of the micropipette correspond to the pressure indicated on the gauge. There remains, however, the consideration of the curved surface of the fluid inside the micropipette. In forcing the fluid through its orifice, the greater pressure is on the concave side of the fluid inside. If the radius of curvature of the fluid inside exceeds a maximum value, an additional amount of pressure must be applied, and flow would result, thereby upsetting hydrostatic conditions. A correction for this was worked out by Mr. Reuben Copperman, of the physics department of the University of Pennsylvania. The correction turned out to be so small as to be entirely negligible. Another objection which could be raised is that an adsorbed surface layer on the walls of the micropipette or attraction of the surface molecules of the walls upon the inclosed fluid could cause a deviation from the laws of viscous flow. Bulkley (1931), working with various liquids in glass capillaries of 5.95-μ internal radius, found that there was no such deviation. With these difficulties solved, it was felt that the method for measuring the pressure acting on the cell membrane was satisfactory. As a matter of fact, Landis (1926) measured the capillary pressure in frog mesentery in essentially the same manner.

Obviously, cell membranes cannot

withstand very high internal pressures. The maximum pressure tolerated by the *Arbacia*-egg membrane is not of the order of atmospheres but rather of the order of $\frac{1}{100}$ atm., and the values for two other marine eggs are similarly low. There can be little doubt that, even with a considerable difference in osmotic pressure across the membrane, as is the case in osmotic swelling in hypotonic solutions, the actual pressures acting on the membrane are very small. If the pressure resistance of the *Arbacia*-egg membrane in 50 per cent sea water is only 2.1 mm. Hg. or 0.0018 atm., then the actual pressure against the membrane must be only a fraction of this value, for the pressure resistance measured here represents a maximum tolerance of pressure before bursting. The fact that dilute sea water lowered the normal resistance of the membrane must be regarded as a complicating factor in the method of Goldforb and Schechter (*op. cit.*), hitherto unnoticed. The initial rise in resistance of both *Arbacia*- and *Chaetopterus*-egg membranes in 80 per cent sea water cannot be explained at this time and requires further study. Possibly, the distilled water used had a metal impurity.

The differences in membrane resistance among the three types of eggs used are relatively slight, considering the fact that they are from three different species. There was no evidence of correlation between membrane resistance and stage of maturation of the egg in any of the three species employed. Of interest, however, is the fact that in the *Chaetopterus* egg[3] there were always two ruptures that could be obtained. Since the first rupture invariably took place at the animal pole, it is clear that this region of the egg surface is a point of weakness. This could be interpreted to mean that the membrane of the *Chaetopterus* egg is physiologically differentiated at the germinal vesicle stage.

The action of the common cations of sea water—sodium, potassium, calcium, and magnesium—on living cells has been intensively studied. Some of their specific effects on cells are well known. Thus, sodium and potassium ions generally increase the permeability of the plasma membrane, while magnesium and calcium produce the opposite effect. For example, McCutcheon and Lucké (1928, 1929) found that sodium and potassium increased the permeability of *Arbacia* eggs to water, while calcium and magnesium decreased it. Both sodium and potassium lowered the resistance of the membrane to internal pressure, while calcium and magnesium increased it. The action of the trivalent aluminum ion on membrane resistance can be attributed to the strong adsorption of this ion by the cell surface. Lucké and McCutcheon (1929) studied the effect of valence of ions on the permeability of the *Arbacia* egg to water. They concluded that positive ions decreased the permeability, the effect being greater, the higher the valence.

From the foregoing, there seems to be a correlation between pressure resistance and permeability of the membrane. This would indicate that the mechanical and structural properties of the membrane, as studied by its pressure resistance and its physiological state, are intimately connected. Some authors attempt to dissociate the two and seek to identify each with a particular chemical constituent of the membrane. Thus, Wolpers (1941) claims that the mechanical and dynamic functions of the erythrocyte membrane are the properties of its protein mesh-

[3] *Chaetopterus* eggs, when shed into sea water, are in the germinal vesicle stage and reach the metaphase of the first maturation division in about 20 minutes. Maturation then ceases until fertilization occurs (Mead, 1898).

work, whereas the regulation of the permeability is attributable to its lipids. The fact that trypsin lowered the resistance of the *Arbacia*-egg membrane to internal pressure suggests that the structural stability of this membrane may well be due, at least in part, to a protein component. This is of interest because our knowledge of the chemistry of cell membranes is very poor, being confined almost exclusively to the erythrocyte ghost membrane (Parpart and Dziemian, 1940). Trypsin does not modify the permeability of the erythrocyte (Ballentine and Parpart, 1940). In view of the modification produced by trypsin on the surface of the *Arbacia* egg, its possible effects on the permeability of that cell should be investigated.

Considering the importance of the biological effects of radiation, the action of radiations on cell membranes has been insufficiently studied. In the studies that have been made on permeability, conflicting results have been obtained. Heilbrunn and Mazia (1936) believe that all kinds of radiation, including ultraviolet, increase cellular permeability. However, Reed (1948) failed to observe an increase in permeability of sea-urchin eggs after exposure to ultraviolet light. Heilbrunn and Daugherty (1933) conclude that ultraviolet light may release calcium ions from the cell surface. In the present investigation, a marked decrease in membrane resistance was noted after exposure to ultraviolet light. As previously noted, excess calcium in both acid and alkaline solutions caused a pronounced increase in resistance. The most likely interpretation of the effect of ultraviolet light on membrane resistance is that the irradiation releases calcium ions from the surface. Accordingly, if this interpretation is correct, it may be said that calcium ion is an important factor contributing to the structural stability of the membrane.

Even less certain than the effects of radiation on permeability is the action of anesthetics on the plasma membrane. Whereas it is generally believed that anesthetics in low concentration cause a decrease in permeability, a critical analysis of the facts does not warrant such a sweeping generalization. For a discussion of this point see Davson and Danielli (1943). Lillie (1918) studied the effect of various fat-solvent anesthetics on the permeability of *Arbacia* eggs to water. These anesthetics did not decrease the permeability when the eggs were placed in dilute sea water but prevented an increase in permeability accompanying fertilization. Heilbrunn (1925) found no decrease in permeability of the same eggs when exposed to dilute ether. Lucké (1931), using urethanes and carbamates, failed to find an increase in permeability of *Arbacia* eggs in dilute sea water. When placed in nonelectrolyte medium, however, a reversible decrease in permeability occurred. Dilute anesthetics either lowered or raised the resistance of the *Arbacia*-egg membrane to internal pressure. In the case of ethyl urethane, the lower concentracion raised the resistance only slightly, while the higher concentration raised it considerably. However, all other anesthetics used lowered the resistance. The evidence, even though it is inconclusive, favors the assumption that anesthetics in low concentration lower the resistance of the cell membrane to internal pressure. A possible interpretation is that the stability of the membrane is lowered by these substances by virtue of their effect on a lipid constituent of the membrane, possibly a protein-lipid complex.

Alkalinity of the medium lowered the resistance of the membrane only slightly. The increase in membrane resistance in

acid media was more pronounced. Apparently, a low pH is toxic to the eggs, and this toxic effect may well be primarily on the cell surface.

The pressure resistance of the cell membrane provides the cell physiologist with a new and direct method of study. The various treatments of the eggs used in this investigation have shown that the cell membrane is an extremely sensitive indicator of environmental changes. If the data obtained from resistance measurements are viewed in the light of permeability studies, a more complete interpretation of the nature of the membrane may be obtained. Undoubtedly, the effect of hypotonic solutions shows that the membrane cannot be exposed to high pressures. Additional evidence is presented to show the importance of calcium ion for the maintenance of the cell membrane. Indirect information concerning the chemical composition and the structure of the membrane is provided by the resistance measurements in the presence of such substances as enzymes and fat solvents.

SUMMARY

1. A method was developed for studying the resistance of cell membranes to internal pressure.

2. The egg-cell membranes of three species of marine invertebrates, *A. punctulata*, *As. vulgaris*, and *C. pergamentaceous*, were found to break when exposed to internal pressures of the order of $\frac{1}{100}$ atm.

3. In hypotonic sea water the resistance of the cell membrane to internal pressure increased with increasing hypotonicity; then, as the sea water was diluted further, the resistance decreased progressively.

4. Ultraviolet radiation and fat-solvent anesthetics decreased the resistance of cell membranes to internal pressure.

5. The divalent cations, calcium and magnesium, increased the pressure resistance of the membrane, while the univalent sodium and potassium ions decreased it.

6. In highly acidified sea water the membrane became very resistant, while at the alkaline extreme of neutrality the membrane became only slightly less resistant.

7. The proteolytic enzyme, trypsin, markedly weakened the membrane. This effect was abolished when the enzyme was heat-inactivated.

8. The discussion of the results obtained was preceded by a justification of the newly developed method for measuring pressure. The method, it was pointed out, is valid only when true hydrostatic conditions are maintained in the system. A further discussion of the method, based on surface chemical considerations, was given.

9. An attempt was made to correlate the pressure resistance of the cell membrane with its permeability. In the cases where certain experimental treatments are known to affect permeability by increasing or decreasing it, the same treatments had a corresponding effect of increasing or decreasing the resistance of cell membranes to internal pressure. This was found to hold true especially for the solutions of cations.

10. The method of measuring pressure resistance of the cell membrane was found to have some value in elucidating the structure and chemical composition of the plasma membrane. The action of fat-solvent anesthetics pointed to the existence of some lipid component in the membrane, possibly a lipoprotein complex. In view of the importance of the calcium ion on the cell surface, it was suggested that this ion might be a struc-

tural component of the membrane. The effect of the proteolytic enzyme, trypsin, in lowering the resistance of the cell membrane to internal pressure strongly points to the existence in the membrane of a protein component.

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CHANGES IN THE DISTRIBUTION OF NITROGEN DURING STARVATION IN THE GRASSHOPPER, CHORTOPHAGA VIRIDIFASCIATA DE GEER

DANIEL LUDWIG

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LUDWIG (1950) reported that during complete starvation, at 25° C. and 82 per cent relative humidity, grasshopper nymphs lost approximately 40 per cent of their original weight. Although under these conditions water was lost at the same rate as body-weight, most of the weight loss was the result of the elimination of metabolic water. The energy used during the initial phases of starvation was obtained chiefly from the oxidation of glycogen and glucose. Fat was the principal source of energy during the latter part of the survival period. The utilization of nitrogenous compounds occurred at a slow, constant rate; the amount of nitrogen lost during complete inanition was only 11 per cent of the

original quantity. Since other constituents were lost at a more rapid rate, the percentage of nitrogen increased from 2.82 in normal to 4.85 in completely starved nymphs. Protein percentages obtained by multiplying these figures by 6.25, the protein conversion factor, increased from 17.63 in normal nymphs, to 30.31 in starved nymphs at the time of death. When these protein percentages were added to the percentage values of all the other determined constituents, the total increased from 97.5 to 105.8 per cent of body-weight. These calculations are obviously incorrect. One error is the assumption that all the nitrogen in the insect is protein nitrogen. It was also suggested that perhaps the conversion factor, 6.25, may not be applicable to the starved insect or that starvation may result in a change in the distribution of nitrogen within the insect, probably in the storage of waste nitrogen.

The present experiments are an attempt to solve this problem by the fractionation of the nitrogenous compounds of normal and of completely starved nymphs.

MATERIAL AND METHODS

Grasshopper nymphs were collected in New York City during the fall of 1949. Their care and experimental treatment preliminary to the chemical studies were essentially the same as in the previous study (Ludwig, 1950).

Nitrogen determinations were made individually by the Kjeldahl procedure on 20 normal nymphs and on 21 others following complete inanition. These determinations were made on the various extracts obtained by the following modification of the method of Hiller, Plazin, and Van Slyke (1948) for the purification of proteins. The insect was weighed, dried under vacuum desiccation at room temperature, then powdered in a 15-cc. centrifuge tube. A solution consisting of 1 cc. distilled water, 4.5 cc. absolute ethyl alcohol, and 4.5 cc. absolute ethyl ether was then added. This solution with the crushed insect was then thoroughly mixed by stirring, and the mixture was allowed to stand, with frequent stirring, for 2 hours. Separation was accomplished by centrifugation, and the supernatant liquid, containing dissolved lipids, decanted into a 100-cc. Kjeldahl digestion flask. The remaining precipitate was washed with another 10-cc. portion of the alcohol-ether solution, separated by centrifugation, and the supernatant liquid added to that already present in the digestion flask. The ether and most of the alcohol were evaporated from the extract by heating the flask over a hot-plate. The remaining extract was allowed to cool, then diluted with 25 cc. of distilled water to prevent the decomposition of the alcohol during the digestion process, and its nitrogen content was determined. The nitrogen obtained by this procedure is lipid nitrogen.

The powder remaining after the removal of the lipids was extracted with 12 cc. of boiling distilled water. The extract was separated by centrifugation and decanted into another 100-cc. digestion flask. This water extraction was repeated twice, each extract being added to that already present in the flask. Its nitrogen content was then determined. This procedure gave values for the water-soluble nitrogen.

The portion of the insect remaining after the removal of its lipids and water-soluble constituents was transferred quantitatively to a 100-cc. digestion flask and its nitrogen determined. The nitrogen contained in this fraction is called "insoluble nitrogen." It includes protein nitrogen and also that present in the chitin of the exoskeleton.

RESULTS

There was very little nitrogen lost during the fractionation process. Total nitrogen values for normal insects obtained by adding together the lipid, soluble, and insoluble fractions averaged approximately 5 per cent less than those calculated by multiplying body-weights by 2.82, the nitrogen percentage.

The results of the nitrogen fractionation studies are given in Table 1. Since it is impossible to make determinations of total nitrogen and to fractionate the nitrogen of the same insect, values for total nitrogen were obtained from the paper by Ludwig (1950). The original weight of the nitrogen, 2.90 mg., was obtained by adding 2.58 mg., the amount remaining in the insect after complete inanition, to 0.32 mg., the amount recovered from the feces eliminated during the starvation period. The table shows that a decrease in lipid nitrogen occurred as a result of starvation, the percentage values for this fraction decreasing from a normal value of 9.4 to 6.3. The difference between these means is more than twelve times its probable error. These figures represent a loss of more than 40 per cent of the lipid nitrogen originally present. The percentage of nitrogen contained in the water-soluble fraction remained approximately constant during starvation. However, because of the loss of body-weight, there was a loss of nitrogen from this fraction which amounted to 8.2 per cent of its original weight. The nitrogen percentage of the insoluble fraction showed a slight increase. However, mean values for normal and for starved insects are not significantly different. The loss of nitrogen from this fraction was 7.9 per cent of its original weight.

The amount of nitrogen lost from each fraction was obtained by multiplying its percentage value by the total weight of the nitrogen and subtracting the amount present at the time of death from that present in normal insects. The total nitrogen lost during complete starvation, obtained by adding together the amount of nitrogen lost from each fraction, was 0.32 mg., which is also the average amount recovered from the feces eliminated during the starvation period.

The figures contained in Table 1 show that 69.7 per cent of the nitrogen in normal nymphs and 72.1 per cent of that in starved nymphs is contained in the insoluble fraction. Since this fraction contains exoskeleton as well as tissue proteins, it contains both chitin and protein nitrogen. Hence, the protein nitrogen must be less than indicated by the percentages given above.

The protein contents of normal and of

TABLE 1

NITROGEN LOST DURING COMPLETE STARVATION

(Values for Total Nitrogen Taken from Table 6, Ludwig, 1950)

Fraction	Normal Per Cent	Original Weight (Mg.)	Final Per Cent	Final Weight (Mg.)	Weight Lost (Mg.)	Per Cent Lost
Total N	.....	2.90	.....	2.58	0.32	.....
Insoluble N	69.7±0.44	2.02	72.1±0.71	1.86	0.16	7.9
Soluble N	20.9±0.36	0.61	21.6±0.62	0.56	0.05	8.2
Lipid N	9.4±0.16	0.27	6.3±0.18	0.16	0.11	40.8
Total	.....	.....	.....	.....	0.32	.....

completely starved insects were recalculated on the assumption that the nitrogen contained in the insoluble fraction is protein nitrogen, using the data from Tables 1 and 4 of Ludwig's 1950 paper, and 6.25 as the protein conversion factor. These recalculated protein percentages are 12.28 and 21.88, respectively. When these new figures are added to the total percentages for the other determined constituents of normal and of starved nymphs, the totals are 92.17 and 97.37 per cent of body-weight, respectively. These figures appear to be more accurate than do those published in the earlier paper.

DISCUSSION

These experiments show that the nitrogen-containing lipids of grasshopper nymphs decrease in amount during starvation and suggest their use as a source of energy. Since the alcohol-ether solution used in the extraction of fats is a solvent for phospholipids, it is likely that a major portion of the nitrogen-containing lipids are phospholipids. Sinclair (1935) divided these compounds into two classes. One class contains the more highly unsaturated fatty acids and is believed to be structural in nature and to enter into the essential makeup of the cell. The other class contains less unsaturated fatty acids and is believed to function in intermediary metabolism. This latter class is presumably utilized in oxidations. In his review article Sinclair (1934) discussed the phospholipid changes which occur during inanition in various tissues of vertebrates. The results are not constant, but decreases have been described in the phospholipid content of skeletal muscle and liver of fasting animals. More recently, MacLachlan, Hodge, Bloor, Welch, Truax, and Taylor (1942) reported a reduction in the weight of the phospholipids from the liver of a mouse starved for 4 days. This reduction amounted to half the original weight of these compounds. During the same time, the weight of the liver decreased by half, so that the phospholipid concentration remained constant.

Holter and Zeuthen (1949) studied the metabolism of the starving amoeba *Chaos chaos*. In this organism the total lipid consumed during starvation may be several times greater than that present as fat globules. Hence, they assumed that protoplasmic lipids (mainly phospholipids) are oxidized. This source of energy may be exhausted before the fat globules are utilized. Their work indicates the possible utilization of structural phospholipids during starvation. In the present work not only was there a decrease in the amount of nitrogen-containing lipids, but there was also a significant decrease in the concentration of these compounds. These observations indicate that, in the grasshopper, structural lipids may also be oxidized during inanition.

The decrease in lipid nitrogen is the only significant change observed in the distribution of nitrogen during starvation in the grasshopper nymph. It results in a slightly greater concentration of nitrogen in the insoluble fraction of the starved insect. There is no evidence for the storage of waste nitrogen as a result of starvation.

The present experiments indicate that the protein conversion factor, 6.25, may be approximately correct for both normal and starved nymphs. Protein concentrations determined by multiplying the nitrogen contained in the insoluble fraction by this factor give results which appear to be reasonably correct. However, since no conversion factors are available for insect protein, it is planned to extend this study to include protein/nitrogen ratios for both normal and starved insects.

This series of experiments suggests a fundamental difference in the metabolism of starvation in the grasshopper and in the mammal. Best and Taylor (1943) give the following account of starvation in mammals: "An animal deprived of food derives energy first from the combustion of its own carbohydrate stores (glycogen). The fat reserves are next drawn upon and finally, after these have been exhausted, tissue protein is broken down, the fatty acid part of the molecule is burned while the nitrogen is excreted in the urine as urea." With regard to nitrogen excretion, they state that the total output of nitrogen in the urine falls for the first day or two of the fast, when the body is subsisting upon its carbohydrate supplies. It then shows a steady rise and usually reaches a maximum about the third or fourth day, and from then on it shows a progressive decline. It shows a pronounced rise shortly before death. The first rise in nitrogen output represents the mobilization of "deposit protein" and the premortal rise indicates the exhaustion of fat stores and the utilization of body protein for energy purposes. In the starving grasshopper nymph, Ludwig (1950) reported that after the first 1 or 2 days, nitrogen was eliminated at a rather slow, constant rate until the last day of survival, when no measurable amount was excreted. Total excreted nitrogen was approximately 11 per cent of the original quantity. It has now been shown that since less than 70 per cent of the nitrogen is present in the insoluble fraction, the loss of protein nitrogen is less than 8 per cent of the original quantity. It has also been found that about one-third of the excreted nitrogen comes from lipids and half from the insoluble fraction, the balance being derived from water-soluble compounds (Table 1). There is no evidence of "deposit protein" and there is no premortal rise in nitrogen excretion. Thus, it appears that, in the grasshopper, body protein cannot serve as an energy reserve.

SUMMARY

1. Determinations were made on the nitrogen contained in the lipid, water-soluble, and water-insoluble fractions of normal grasshopper nymphs and of others following complete inanition.

2. Starvation resulted in a significant decrease in the nitrogen-containing lipids, the amount of nitrogen lost from this fraction being more than 40 per cent of the original quantity. It is suggested that nitrogen-containing lipids, probably phospholipids, serve as an important source of energy in this insect.

3. No significant changes were observed in the nitrogen contained in the soluble and insoluble fractions as a result of starvation. The nitrogen lost from each of these fractions was approximately 8 per cent of the original quantity.

4. The total nitrogen lost during complete starvation, obtained by adding together the amount of nitrogen lost from each fraction, was 0.32 mg., which is also the average amount recovered from the feces eliminated during the starvation period.

5. There is no evidence for the storage of waste nitrogen during starvation.

6. The protein conversion factor, 6.25, appears to give satisfactory protein percentages for both normal and starved insects when multiplied by the nitrogen percentages of the water-insoluble fraction of each group.

7. During inanition, the grasshopper nymphs do not appear able to use body protein as an energy reserve.

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SYNTHESIS OF VITAMINS OF THE B-COMPLEX BY CHILOMONAS PARAMECIUM

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GROWTH of the colorless plant-like flagellate, *Chilomonas paramecium*, in media composed of mixtures of inorganic salts, with or without an organic carbon source,[1] has been thought to presuppose an ability of the organism to synthesize known growth factors if it is to carry on metabolism by conventional means. The first conclusive proof for such a synthesizing ability was provided by Hutchens *et al.* (1941) with their demonstration that *C. paramecium* produces diphosphopyridine nucleotide and nicotinic acid in an acetate and inorganic salt medium supplemented with thiamine. Additional suggestive information is found in observations that vitamins of the B-complex stimulate growth of *C. paramecium*. For instance, Lwoff and Dusi (1938) obtained maximum growth in an acetate and ammonium-N medium supplemented with iron and thiamine. This observation was confirmed by Hutchens (1940*a*). Pace (1947) reported stimulatory effects of riboflavin, nicotinic acid, and pyridoxine, as well as thiamine. Cosgrove (1949) obtained massive populations by using ethanol as a carbon source and by supplementing the medium with a mixture of the vitamins which Pace found to be stimulatory.

The rather general assumption that *C. paramecium* possesses the ability to synthesize vitamins of the B-complex, at least on a minimal level consistent with normal growth in simple media, and the absence of any definite evidence other than that of Hutchens *et al.* (1941) suggested the present investigation of the problem by the use of microbiological assays.

The writer would like to express his gratitude to Dr. R. P. Hall and Dr. William B. Cosgrove for their advice, interest, and encouragement in this investigation.

[1] Such results have been reported by Bowen (1940), Burrows (1938), Cosgrove (1950), Hutchens (1940*a* and *b*, 1948), Hutchens *et al.* (1941, 1948), Mast and Pace (1932*a* and *b*, 1933, 1935, 1936, 1937, 1938*a* and *b*, 1939, 1942, 1946), Mast, Pace, and Mast (1936), Pace (1941, 1944, 1947, 1948), Smith (1940), and Sullivan (1947, 1949).

MATERIAL AND METHODS

For all assays described below, *C. paramecium* (a Pringsheim strain obtained from Dr. R. P. Hall) was grown in the following medium, a modification of that used by Hutchens *et al.* (1941):

$CH_3COO\,Na \cdot 3H_2O$	2.5	gm.
NH_4Cl	0.45	gm.
$(NH_4)_2SO_4$	0.10	gm.
$K_2HPO_4 \cdot 3H_2O$	1.95	gm.
$MgCl_2 \cdot 6H_2O$	0.02	gm.
$CaCl_2 \cdot 2H_2O$	0.015	gm.
$FeCl_3 \cdot 6H_2O$	0.003	gm.
Thiamine	10.0	μg
Distilled water	1.0	liter*

* pH of medium adjusted to 6.0 with 0.1N NaOH before sterilization.

The flagellates were maintained in this medium for 10–25 serial transfers before use for assays. All glassware utilized in preparation of the medium or for cultures of *C. paramecium* was Pyrex, with the exception of pipettes and glass spatulas, and was submitted to a rigorous cleaning technique. The procedure included: washing with hot Alconox solution, rinsing with hot tap water, immersion in chromate–sulfuric-acid cleaning solution for 12 hours, rinsing six times with hot distilled water and four times with hot Pyrex-redistilled water, and steaming over Pyrex-redistilled water. Reagents used in preparation of the medium were analytical-grade products of Baker, Eimer and Amend, Mallinckrodt, and Merck, and the water was redistilled in Pyrex. The use of cotton plugs for culture flasks was obviated by following Cosgrove's (1950) technique. Every reasonable care was exercised to prevent introduction of extraneous material into the culture medium during preparation, inoculation, and incubation. Steam was passed through the autoclave for 30 minutes before use. Immediately after preparation, the medium was sterilized at a pressure of 15 pounds and then allowed to cool before removal from the autoclave.

The bacteria used as assay organisms, *Leuconostoc mesenteroides* (Strain 535) and *Lactobacillus casei*, were obtained through the courtesy of Dr. Carl S. Pederson and Nutritional Biochemicals, respectively. Amino acids, purines, sugars, and vitamins used in basal media for the assay organisms were products of Difco, Eimer and Amend, General Biochemicals, Hoffman-LaRoche, Lederle, Merck, and Nutritional Biochemicals. Acid casein hydrolysate was obtained from General Biochemicals and enzymatic casein hydrolysate from Nutritional Biochemicals. Inorganic reagents were the same as those used for *C. paramecium*.

In providing material for assays, *C. paramecium* was grown in 250-ml. flasks containing 100 ml. of the standard medium and covered with 50-ml. beakers. Each flask received a 5.0-ml. inoculum from a 48-hour stock culture and was incubated in darkness at 25° C. until the pH reached 7.5. At this point, the population appeared to be at or near the maximum and to be composed almost solely of intact living organisms. The presence of contaminating microörganisms in a culture would necessarily rule it out for assay purposes; hence contamination checks were made by plating out samples on the acetate and ammonium-N medium plus agar (Difco's Bacto-agar), and on yeast-dextrose agar. In addition, stained smears were examined. A sample of the culture was then withdrawn for counting (hemocytometer method), and the remaining volume was centrifuged at 200–300 g. for 10 minutes. The supernatant liquid was discarded, and the concentrated flagellates were submitted to an appropriate extraction technique. For the assay of nicotinic acid, the flagellates were extracted according to the method used by Krehl *et al.* (1943) and further

described by Johnson (1945). Extraction for pyridoxal was carried out by the method of Rabinowitz and Snell (1947*b*) for liberation of vitamin B_6 from natural materials. The extraction technique for riboflavin was that recommended by Strong (1947).

All assays were set up in Pyrex culture tubes of approximately the same bore, plugged with Johnson's bleached absorbent cotton. Titrimetric measurements were made with a Beckman Acidimeter and turbidimetric measurements with a Coleman Junior spectrophotometer. Extracts were assayed for nicotinic acid by a modification of Johnson's (1945) technique, using *Le. mesenteroides*. Changes in the original procedure involved the addition of enzymatic casein hydrolysate (10.0 gm/liter of basal medium), turbidimetric measurement of the growth response, and pH adjustment of the basal medium with KOH (MacLeod and Snell, 1948). The standard nicotinic acid solution was prepared according to Snell's (1947) recommendations. After incubation for 72 hours, all assay cultures were steamed at 100° C. for 10 minutes and were then cooled to room temperature before measurement of growth (spectrophotometer setting—660 mμ.) Pyridoxal was assayed with *La. casei* by the method of Rabinowitz *et al.* (1948). Riboflavin was assayed with *La. casei* by the method of Snell and Strong (1939) in its latest form (Strong, 1947), with the exception that the basal medium was a commercial preparation, Basal Medium Stock Solution (for microbiological riboflavin assay), obtained from General Biochemicals.

In each assay the concentration of vitamin/ml of extract was calculated by Snell's (1947) method, and the concentration of vitamin extracted from a given number of flagellates was determined from the total volume of extract, from the population (flagellates/ml) at the time of centrifugation, and from the volume of culture fluid centrifuged. The inadequacy of expressing the quantity of vitamin in terms of number of flagellates is fully recognized. Dry weights also would have little meaning because *C. paramecium* stores a tremendous amount of carbohydrate reserve in the form of large granules. Vitamin-containing protoplasm could be measured adequately only by such a method as total nitrogen determination.

EXPERIMENTAL RESULTS

Nicotinic acid.—A typical standard curve for the growth response of *Le. mesenteroides* to nicotinic acid and a corresponding curve for the response to graded amounts of the *Chilomonas* extract are shown in Figure 1. From the assay data it was calculated that 2.4 μg. of nicotinic acid were extracted from the flagellates contained in 94 ml. of culture medium. The population at the time of extraction was 23.8×10^4 (±2,000) flagellates/ml. The concentration of nicotinic acid in the extract was 0.032 μg/ml and the total volume of the extract was 75 ml. The calculated amounts of nicotinic acid extracted in other assays ranged from 1.25 to 2.76 μg. for 40–110 ml. of culture with populations at approximately the same level as that mentioned above.

In these assays for nicotinic acid, enzymatic casein hydrolysate was added to the basal medium of Johnson (1945) in an attempt to shorten the incubation period necessary for turbidimetric measurements (Roberts and Snell, 1946). An increase in rate of growth was noted, and measurement was feasible after 36 hours. It was found, however, that measurements at this time gave excessively high values for nicotinic acid in the extract samples. By extending the growth period to 72 hours, as recommended by Johnson

(1945) for titrimetric measurement, this effect was eliminated. A similar observation was noted by Rabinowitz and Snell (1947*a*) in assaying vitamin B_6 in yeast extract with *Streptococcus faecalis*.

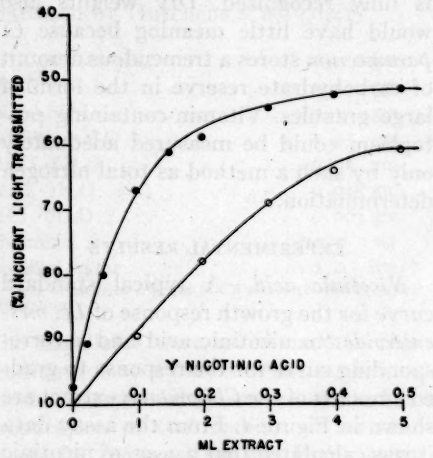

FIG. 1.—Growth response of *Leuconostoc mesenteroides* to nicotinic acid standards (*solid circles*) and to *Chilomonas* extract samples (*open circles*).

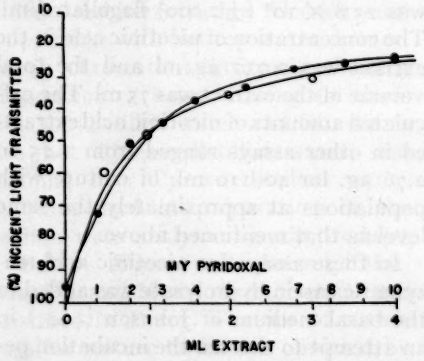

FIG. 2.—Growth response of *Lactobacillus casei* to pyridoxal standards (*solid circles*) and to *Chilomonas* extract samples (*open circles*).

Pyridoxal.—A standard curve for growth response of *La. casei* to pyridoxal and one for the response to *Chilomonas* extract are shown in Figure 2. The data indicate a total of 0.4 μg. of pyridoxal extracted from the flagellates contained in a 96-ml. volume of culture. The population at the time of extraction was 23.8×10^4 (±2,000) flagellates/ml, and the total volume of the extract was 181 ml. The concentration of pyridoxal in the extract was 0.00221 μg/ml. The calculated amounts of pyridoxal extracted in other assays ranged from 0.52 to 0.64 μg. for 125–65 ml. of culture with populations at approximately the same level as that mentioned above.

Riboflavin.—Curves for the response of *La. casei* to riboflavin and *Chilomonas* extract are reproduced in Figure 3. A

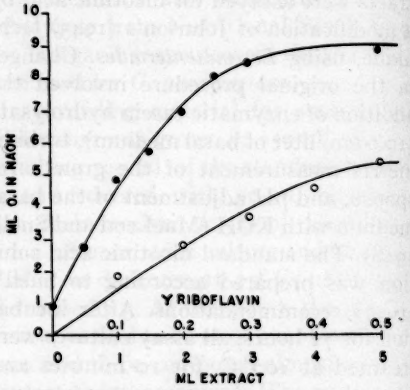

FIG. 3.—Growth response of *Lactobacillus casei* to riboflavin standards (*solid circles*) and to *Chilomonas* extract samples (*open circles*).

total of 0.625 μg. of riboflavin was extracted from the flagellates in a 190-ml. volume of culture, the density of population being 24.9×10^4 (±2,000) flagellates/ml. The concentration of riboflavin in the extract was 0.025 μg/ml, and the total volume of the extract was 25 ml. Data from other assays gave values of 0.34 and 0.701 μg. of riboflavin extracted from the flagellates contained in 100- and 200-ml. volumes of culture, respectively, with population levels approximately equivalent to that mentioned above.

Photolyzed aliquots of the *Chilomonas* extract also were assayed to substantiate

the specificity of the growth response of *La. casei*. Photolysis was carried out by exposing the extract, at pH 7.0, to the illumination of a 100-watt light bulb at a distance of 25 cm. for 24 hours. The photolyzed extract failed to sustain growth of *La. casei*.

Recovery experiments.—In order to determine whether the responses of assay organisms to graded amounts of the *Chilomonas* extracts could be attributed solely to the presence of the vitamins concerned or in part to other stimulatory or inhibitory substances in the extracts, recovery experiments were carried out for each vitamin (Table 1). A measured amount of the vitamin in question was added to duplicate tubes containing extract samples on at least four assay levels. The percentage recovery of added vitamin was determined by comparing the values obtained in the standards and extract samples for each equivalent level of concentration. Recovery values differing by no more than ±10 per cent from the theoretical are considered proof that the assay in question is valid. These recovery checks were used solely to determine the presence or absence of "drift" in the assays, since it was felt that previous results (Johnson, 1945; Krehl *et al.*, 1943; Rabinowitz *et al.*, 1948; Sarett, 1945; Snell and Strong, 1939; Snell and Wright, 1941) with nicotinic acid, riboflavin, and pyridoxal have demonstrated that the extraction techniques employed do not have destructive effects on these vitamins in natural materials.

TABLE 1

RECOVERIES OF VITAMINS ADDED TO EXTRACT SAMPLES

Vitamins Added	Vitamins Found	Recovery (Per Cent)
μG	Nicotinic Acid	
0	0.126	
0.1	0.23	101
0	0.093	
0.1	0.189	97
0	0.059	
0.1	0.16	100
0	0.029	
0.1	0.142	110
MμG	Pyridoxal	
0	2.4	
1.0	3.3	97
0	4.4	
1.0	5.0	93
0	5.9	
1.0	7.38	107
0	9.5	
1.0	10.32	98
μG	Riboflavin	
0	0.055	
0.1	0.172	110
0	0.08	
0.1	0.195	108
0	0.12	
0.1	0.23	105
0	0.145	
0.1	0.26	106

DISCUSSION

In view of the recent withdrawal of earlier claims for *Tetrahymena geleii* (Kidder and Dewey, 1949*a* and *b*), *C. paramecium* now appears to be the only protozoan species for which synthesis of B-complex vitamins has been clearly demonstrated.

The earlier observations of Hutchens *et al.* (1941), that *C. paramecium* synthesizes nicotinic acid, have been confirmed by the present microbiological assays. *Le. mesenteroides* responds to nicotinic acid but not to nicotinamide, except in extremely high concentrations, or to kynurenine and hydroxyanthranilic acid, intermediates in the conversion of tryptophane to nicotinic acid by *Neurospora* (Volcani and Snell, 1948). Since nicotinamide was hydrolyzed completely to nicotinic acid (Johnson, 1945) in the ex-

traction technique employed, the values reported above represent "total" nicotinic acid. It may be noted that Leonian and Lilly (1945) report identical values (± 10 per cent) for the nicotinic-acid content of a yeast sample assayed with *Lactobacillus arabinosus*, *La. casei*, *Le. mesenteroides*, and *Zygosaccharomyces marxianus;* and Snell (1947) points out that the results obtained with *La. arabinosus* check well with those obtained with the cyanogen bromide technique, which was used by Hutchens *et al.* (1941).

Results reported for pyridoxal represent the concentration of that component of vitamin B_6 and no other. Rabinowitz and Snell (1948) report: "At high levels, addition of either pyridoxine or pyridoxamine to the medium permits growth of *La. casei;* they are less than 0.001 as active as pyridoxal for this organism, however, and their contribution to the analytical values found for natural materials are entirely negligible (Snell and Rennefeld, 1945)." In addition, the loss of pyridoxal by transamination reaction with amino acids does not occur under the hydrolytic conditions employed in the extraction or during the assay (Rabinowitz *et al.*, 1948).

The validity of the values obtained for riboflavin is supported by the fact that the Snell and Strong method is the oldest and probably the best-established microbiological assay. Comparison of results obtained with it and with biological and fluorimetric methods shows good agreement (Emmett *et al.*, 1941).

It is obvious that the present data will not permit accurate estimation of the vitamins synthesized by a given number of flagellates. In addition to the unavoidable errors of counting methods, a range of ± 10 per cent is to be expected in the assay techniques. Furthermore, it is not certain that the methods of extraction effect quantitative removal of vitamins from the flagellates or that individual flagellates from populations of somewhat different densities contain identical quantities of a given vitamin. Consequently, the writer feels that such quantitative interpretations as amount of vitamin per million flagellates should be delayed until more is known about this phase of flagellate metabolism.

The knowledge that *C. paramecium* possesses the ability to synthesize nicotinic acid, pyridoxal, and riboflavin provides an insight into some of the metabolic activities which the organism may be expected to carry on, since the physiologically active forms of these vitamins have been described as cofactors for a wide variety of cellular reactions. In addition, the results demonstrate a number of chemical linkages and configurations that the flagellate is capable of synthesizing from relatively simple materials.

SUMMARY

1. The observations of Hutchens *et al.* (1941) that *C. paramecium* synthesizes nicotinic acid in an acetate and ammonium-N medium have been confirmed by microbiological assays.

2. Synthesis of pyridoxal and riboflavin, under comparable conditions, has been demonstrated by similar techniques.

3. Some quantitative data are presented.

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WATER AND SOLID CONTENT OF ENDOCRINE ORGANS OF RATS FOLLOWING PROLONGED INJECTIONS OF TESTOSTERONE PROPIONATE[1,2]

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PROLONGED administration of various androgens, particularly testosterone propionate, which is of immediate interest, into normal male animals shows that there is a decrease in the wet weight of testes (Moore and Price, 1937) and thymi, but no change in the wet weights of adrenals, thyroids, and hypophyses (Korenchevsky and Hall, 1939). Previous studies, to be discussed more appropriately elsewhere, have been limited either to histological interpretations or to determinations of changes in their wet weights or to both. Determinations have not been made of dry weights of the various endocrine organs following prolonged injections of androgens into normal animals.

The present study was undertaken to determine the action of doses of testosterone propionate lighter than hitherto generally reported, i.e, more physiological than pharmacological, and the effect of these doses on changes in the water and solid content of these organs.

MATERIAL AND METHODS

Young male white rats were divided into three groups (see Table 1, first and second cols.): (A) the controls, and two groups (B) and (C) which received, respectively, subcutaneous injections either of 1 mg. ("light" dosage) or of 5 mg. ("heavy" dosage) of testosterone propionate (Ciba) in sesame oil per kilogram of body-weight, three times per week, during a period of 5–6 weeks.

Injections were begun when the rats weighed between 20 and 30 gm. The rats were kept under the usual laboratory conditions, at a constant temperature (82° ± 1° F.); they were fed Purina Dog Chow and tap water *ad libitum*. All animals were weighed once a week. Finally, they were starved overnight (18–22 hours), were weighed, sacrificed, and measured from tip of snout to base of

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tail (body-length). The desired organs (adrenals, hypophyses, testes, thymi, and parathyroids-thyroids) were rapidly, but carefully, removed and immediately weighed to the nearest 0.1 mg. with a torsion balance. The wet weighings were performed by one person, to assure a more constant procedure for the removal of any excess fluid and to facilitate greater rapidity in manipulations in order to prevent undue drying. The organs were then placed in individual vials and were dried overnight (18–22 hours) at 105° C. This procedure gave data for the respective wet and dry weights.

RESULTS

The results for the studies of body-weights, body-lengths, and wet- and dry-weight analyses of adrenals, hypophyses, testes, thymi, and thyroids-parathyroids of normal male rats injected either with "light" or with "heavy" dosage of testosterone propionate are summarized on the basis of their respective mean weights in Table 1. The results of statistical analyses of the differences between the various pairs of means are recorded in the lower portion of the table under "*t*-value." For any given statistical comparison the respective degrees of freedom may be deduced by noting in the table the number of animals employed.

The data show that testosterone propionate either in "light" or in "heavy" dosage has no significant effect on body-weights or body-lengths nor on wet or on dry weights of the thyroid-parathyroids. Statistical significance is found for the following organ weights: for wet-weight determination—"light" dosage produces a decrease in adrenals, hypophyses, and testes; "heavy" dosage causes a decrease in hypophyses, testes, and thymi. For dry-weight studies—"light" dosage gives a decrease in adrenals; "heavy" dosage shows a decrease in hypophyses, testes, and thymi. It follows as a corollary to the foregoing analyses that for both wet- and dry-weight studies "light" dosage is without significance on the thymi and "heavy" dosage is ineffective on the adrenals. Further, though wet weights of hypophyses and testes are significantly affected by the use of "light" dosages of testosterone propionate, their dry weights are not.

DISCUSSION

BODY-LENGTH AND WEIGHT

Experiments which treat of injections of androgens into normal males over long periods of time are not numerous. Rubinstein, Kurland, and Goodwin (1939) and Kochakian (1940), daily injecting 1 mg. and either 0.2 or 0.4 mg. of testosterone propionate into young male rats and mice respectively, found a decrease in body-lengths and weights. Rubinstein and Solomon (1940), on the other hand, employing a lighter daily dose (0.05 mg.) found a significant increase. Bottomley and Folley (1938) daily injected 1.2 mg. of various androgens into young male guinea pigs and found, after 28 days, no effect on body growth, with the exception of 17-methyl testosterone, which gave a "significantly greater increase than that of the controls." Prior to these reports, McEuen, Selye, and Collip (1937) failed to demonstrate any inhibition of somatic growth in young rats receiving daily injections of 0.2 mg. of testosterone propionate but found, instead, a "suggestion of growth stimulation, at least in the normal female rat." The results reported here are similar to those of the last group of workers in that, although not statistically significant, they suggest a consistent trend toward an increase in the body-lengths and weights. Hitherto the weakest daily dose of testosterone propionate reported (Rubinstein and Solomon, 1940) falls between the "light" and "heavy"

TABLE 1

MEANS OF BODY WEIGHTS AND LENGTHS, AND OF WET AND DRY WEIGHTS OF ADRENALS, HYPOTHESES, TESTES, THYMI, AND THYROIDS-PARATHYROIDS OF NORMAL RATS FOLLOWING PROLONGED INJECTIONS OF TESTOSTERONE PROPIONATE OF INDICATED DOSAGES*

Rats ♂, 20–30 gm.		Groups	Wet or Dry	Body-Weight (Gm.)	Body-Length (Mm.)	Adrenals (Mg.)	Hypophyses (Mg.)	Testes (Mg.)	Thymi (Mg.)	Thyroids-Parathyroids (Mg.)
Controls		A	W	118.8±2.7 (12.9)	175.1±1.8 (8.6)	18.4±0.5 (2.5) 23	5.6±0.2 (0.7) 16	1908.8±70.9 (274.8) 15	361.9±14.4 (49.9) 12	12.0±0.7 (3.1) 22
			D	.....	.....	5.9±0.2 (0.7) 11	1.9±0.2 (0.6) 10	230.5±11.8 (37.4) 10	72.8± 4.8 (15.2) 10	4.0±0.3 (1.0) 11
Dosage-strength Testosterone propionate	Light, 1 mg.	B	W	122.5±3.3 (15.0)	177.0±1.8 (8.4)	16.3±0.6 (2.7) 21	4.7±0.3 (1.0) 14	1632.0±83.2 (288.5) 12	334.6±24.3 (84.3) 12	12.0±0.7 (7.4) 12
			D	.....	.....	5.0±0.2 (0.8) 16	1.6±0.1 (0.4) 14	203.6 ±15.1 (37.0) 6	74.0±5.4 (13.1) 6	4.0±0.4 (1.0) 6
Mg/kg/ 3×/wk/ 6 wk	Heavy, 5 mg.	C	W	124.0±3.0 (10.3)	179.0±1.8 (6.2)	17.4±0.9 (2.9) 12	5.0±0.2 (0.7) 10	1236.4±97.8 (339.3) 12	304.2±18.4 (63.7) 12	11.8±0.8 (2.6) 12
			D	.....	.....	5.5±0.3 (0.7) 6	1.4±0.06 (0.2) 6	170.6±22.1 (54.2) 6	58.0± 4.2 (10.2) 6	3.8±0.2 (0.6) 6
Statistical comparison of significance ("*t*-values")		A-B	W	NS 0.868	NS 0.748	HS 3.72	S 2.50	S 2.53	NS 0.97	NS ∞
			D	.....	.....	S 3.18	NS 1.34	NS 1.40	NS 1.68	NS 0
		A-C	W	NS 0.782	NS 1.531	NS 0.76	S 2.12	HS 5.57	S 2.46	NS 0.19
			D	.....	.....	NS 1.11	S 2.54	S 2.39	S 2.32	NS 0.55

"W" = wet weight; "D" = dry weight.

A box contains a given mean ± its standard error and, immediately underneath, ± its standard deviation in parentheses. The number in the lower right corner = number of animals (variates) employed.

"*t*-value" = difference between two means/estimated standard error of this difference.

Statistically: "NS" = not significant ($P > 0.05$); "S" = significant ($P = 0.05$–0.01); "HS" = highly significant ($P < 0.01$).

dosages employed here. It is suggestive to conclude that relatively light doses of testosterone tend to increase, while heavy doses decrease, normal body growth.

THYROID-PARATHYROID

On the basis of the present technique, the thyroids-parathyroids when excised as a unit showed no statistically significant change either in wet or in dry weights after administration either of "light" or of "heavy" dosage of testosterone propionate. Although these individual dosages are considerably less than hitherto reported for thyroid studies, the present results obtained are in agreement with the work of Bottomley and Folley (1938). These workers found no change in the wet weights of the thyroids of guinea pigs after treatment with daily doses of 2 mg. of testosterone propionate.

The present results may be due to subthreshold dosage, since Carrière, Morel, and Gineste (1938), injecting 5 mg. of testosterone propionate every second day for 8–20 days, reported an activation of the thyroids of guinea pigs; and Selye (1939), employing 5 mg. per day for 20 days, found that "the thyroids of the testosterone-treated mice are larger than those of the controls."

THYMUS

The administration of "light" dosage of testosterone propionate to normal male rats had no significant action either on wet or on dry weights of thymi. In view of subsequent results, this effect must be interpreted as due to the use of subthreshold dosages. "Heavy" dosages of testosterone propionate showed a significant decrease both in wet and in dry weights of thymi. The results on wet weights are in accord with the literature, which repeatedly demonstrates that the administration of various androgens causes a decrease in the wet weight of the thymi of male animals (Korenchevsky, Dennison, and Kohn-Speyer, 1932; Korenchevsky and Dennison, 1934; Korenchevsky, Dennis, and Eldridge, 1937; Schacher, Browne, and Selye, 1937; Korenchevsky, Hall, and Ross, 1939; Reinhardt and Wainman, 1942).

A comparison of the wet and dry weights of these thymi shows that the protoplasmic solids are decreased to a greater degree than is tissue and protoplasmic water. This is contrary to the results observed for the testes, in which loss of water exceeds loss of solids.

TESTIS

Moore and Price (1932, 1938), McEuen, Selye, and Collip (1937), Bottomley and Folley (1938), and Korenchevsky and Hall (1939) showed that injections of androgens, particularly testosterone propionate, into normal rats induce atrophic changes of the testes as interpreted on histological and on wet-weight bases. The present studies extend the foregoing observations on wet weights by showing that, with the use of "light" dosage, the observed atrophy of testes is due only to a loss of water.

ADRENAL

"Heavy" dosages of testosterone propionate administered to normal male rats altered neither the wet nor the dry weights of the adrenals. These results are in agreement with the literature (McEuen, Selye, and Collip, 1937; Bottomley and Folley, 1938; and Korenchevsky and Hall, 1939), which treats only of the wet weights of this gland. Contrarily, "light" dosages of testosterone propionate gave statistically significant decreases both in wet and in dry weights of the adrenals. Korenchevsky and Hall (1939) found that, of the various androgens studied,

only androsterone produced a change (decrease) in the wet weights of the adrenals of the male rat. An earlier report (Korenchevsky, Dennison, and Hall, 1937) showed a similar response on daily injections of 0.5 mg. of testosterone propionate into normal female rats. This led Korenchevsky and Eldridge (1937) to conclude that "perhaps it is not accidental that a weaker effect with larger dose was also seen on the injection of testosterone propionate into ovariectomized rats." The present results are apparently explainable on the basis of the low concentration of testosterone propionate injected. The "light" dosage used here is quantitatively equivalent to a daily dose of slightly less than 0.04 mg. of testosterone propionate. Korenchevsky and Hall's (1939) results for androsterone may be attributable to a quantitative interpretation; although they daily injected higher concentrations than employed here, it is well known that androsterone has a lower biological potency per unit weight than does testosterone or its ester (Bottomley and Folley, 1938). Thus, apparently, the administration of low concentrations of testosterone propionate is more effective in producing a change in the wet and dry weights of the adrenal, though higher concentrations are ineffective.

HYPOPHYSIS

"Light" dosages of testosterone propionate caused a statistically significant decrease in wet weight, but "heavy" dosages produced a significant decrease not only in wet but also in dry weights of the hypophyses. These results are at variance with the literature treating of the action of androgens on the wet weights of this gland. McEuen, Selye, and Collip (1937), Korenchevsky and Hall (1937), and Bottomley and Folley (1938), using stronger doses than those employed here, could find no change in the wet weights of the hypophyses of rats and guinea pigs. The interpretation of these results, like those relating to "light" dosages of testosterone propionate on adrenal weights, must be sought on the basis of the relatively low concentration of the hormone administered.

GENERAL

Two characteristic reactions to injection of testosterone propionate, based on the technique employed in these experiments, are: (1) if there is a change either in wet or in dry weights of the organs studied, the change is always one of decrease (cf. adrenals, hypophyses, testes, and thymi); (2) "light" dosage of testosterone propionate causes a statistically significant decrease in water content prior to a decrease in tissue solids (cf. hypophyses, testes, and possibly adrenals).

Concerning the first generalization, the question arises as to whether the observed decrease in dry organ weights is due to the action of testosterone directly on the organs in question or indirectly through the mediation of the various trophins of the pituitary. Since the work of Moore and Price (1930, 1932, 1937), Hertz and Myers (1937), Wolfe and Hamilton (1938), and Cutuly and Cutuly (1938), the conclusion is accepted that increasing the concentration of gonadal hormone results in the inhibition of the hypophyseal production of gonadotrophin. Indeed, it has been suggested that gonadal hormone (estrogens and androgens) may affect the production of somatotrophin (Rubinstein, Kurland, and Goodwin, 1939). Should the latter prove true, then changes in adrenal weights might also be interpreted as due to an imbalance in the production of corticotrophin. The thymus, a question-

able endocrine organ, does not lend itself to the foregoing interpretation. It appears, rather, that testosterone and, for that matter, steroid hormones in general exert their action directly on the thymus (Selye, 1947, p. 683).

Concerning the second generalization, it may be that testosterone acts in some as yet unknown manner, and possibly in low concentrations as well, to cause, directly or indirectly, a shift in tissue water. That certain related steroids, particularly the adrenocortical hormone, may exert this effect is well known (Gonzalez and Angerer, 1947). Indeed, androgens resemble adrenocortical hormone in its ability to influence water shift in certain tissues of the body (Burrows, 1945, p. 236). Whether a direct action is the sole cause or whether the inhibition of hypophyseal activity with its concomitant decrease in the secretion of various "trophins" leads to a decreased activity and a water loss on the part of the various organs still needs solution. It is to be concluded that low levels of testosterone in the normal rat tend to cause a decrease in water content of various endocrine organs prior to an eventual decrease in protoplasmic solids. That a decrease in protoplasmic water is correlated with a decrease in function is well recognized (Heilbrunn, 1943, p. 19).

SUMMARY

Young male rats were divided into three groups: the controls (group A) and two groups which received, respectively, injection of 1 mg. ("light" dosage, group B) and 5 mg. ("heavy" dosage, group C) of testosterone propionate per kilogram of body-weight, three times a week for 5–6 weeks. The following results, which have been treated statistically, were obtained (see Table 1).

1. Neither "light" nor "heavy" dosage produced any significant change in the wet or the dry weights of the thyroids-parathyroids or in body-lengths and weights.

2. "Light" dosage caused a significant decrease in the wet weights of the hypophyses and testes but not in their dry weights, while "heavy" dosage caused a significant decrease both in wet and in dry weights.

3. Although "light" dosage showed no change in the thymus weights, possibly because of its subthreshold concentration, the "heavy" dosage produced a decrease both in wet and in dry weights of this organ.

4. "Light" dosage showed a highly significant decrease both in the wet and in the dry weights of the adrenals, but "heavy" dosage produced no significant change in either of these.

5. The results suggest that, prior to any decrease in protoplasmic solids, these is a decrease in the water content of various endocrine organs following prolonged treatment with testosterone propionate.

6. These results are discussed in the light of the existing literature and are interpreted on the basis of an indirect action mediated through the pituitary, although a possible direct action of testosterone may influence the observed water shift.

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OXYGEN CONSUMPTION OF TESTES FROM ADRENALECTOMIZED AND NORMAL RATS AFTER PROLONGED TREATMENT WITH TESTOSTERONE PROPIONATE[1]

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ALTHOUGH many reports exist concerning the action of androgens on wet weights and on histological changes in various endocrine organs (see review of Burrows, 1945, p. 232), Angerer and Gonzalez (1950) concluded, relative to the present study, that "light" dosage (1 mg/kg body-weight injected 3 ×/week for 5–6 weeks inclusive) initially produce a decrease in wet weights of hypophyses and testes and that "heavy" dosage (5 mg/kg) cause a decrease in dry, as well as a greater decrease in wet, weights of these organs. Third, that wet- and dry-weight changes of the adrenals are more readily induced by lower than by higher concentrations of testosterone propionate. These workers, in order to account for the decrease in water and protoplasmic solids of the organs in question, emphasized the classic interpretation of Moore and Price (1930, 1932) for testicular involvement and suggested a hypophyseal involvement for the adrenal, as was previously suggested by Rubinstein, Kurland, and Goodwin (1939) for changes in body-weights following injections of androgen and estrogens, respectively. Of course, a direct action, particularly for water shift, may not be out of the question (Burrows, 1945, p. 236).

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The present study was undertaken to determine from a metabolic viewpoint the effect of varying the balance between certain steroid hormones as it affects one member of the adrenal-gonadal-hypophyseal interrelationship, namely, the testes.

MATERIAL AND METHODS

The oxygen uptake of testes was determined by the Warburg manometric technique. The manometers were oscillated through an amplitude of 3.9 cm., at a rate of 62 cycles per minute. A respiration flask contained 0.2 ml. of 10 per cent NaOH in the central well, 2 ml. of oxygenated Krebs's solution (Umbreit, Burris, and Stauffer, 1949, p. 119), plus 0.5 ml. of testicular mixture in the main chamber. All Krebs's solutions were buffered at pH 7.4 with Na_2HPO_4. The manometers were filled with oxygen and equilibrated at 37°.5 ± 0°.01 C. for 15 minutes.

White rats weighing between 20 and 30 gm. were fed Purina Dog Chow and were kept under laboratory conditions at 78° ± 1° F. The rats were divided into six experimental groups: (A) normal (unoperated) rats; (B) adrenalectomized rats, two groups of normal rats which received subcutaneous injections of either (C) 1 mg. ("light" dosage) or (D) 5 mg. ("heavy" dosage) of testosterone propionate (Perandren, Ciba) in sesame oil/kg of body-weight injected 3 ×/week for 5–6 weeks inclusive; and two groups, E and F, which were treated in the same

manner as were groups C and D, respectively, but were bilaterally adrenalectomized 5 days prior to use.

Tissue was obtained from rats which weighed between 80 and 120 gm. The rats were starved from 18 to 21 hours prior to sacrifice. Each experiment was based on five to six right testes, from which the tunicae and epididymides had been removed. The testes were minced and pooled. Samples of 200 mg. of the pooled testicular mince were rapidly weighed with a torsion balance and were mixed with 0.5 ml. Krebs's solution. To each of six respiration flasks was added 0.5 ml. of the testicular-Krebs's mixture. Aliquot samples of this mixture were placed in individual weighing flasks, and a similar number of samples was prepared with distilled water instead of Krebs's solution. These weighing flasks were dried overnight (17–21 hours) at 105°–110° C. From these latter samples were obtained the tissue dry weights. The oxygen consumption for the testes of the various groups of rats is expressed as Q_{O_2}, i.e., cubic millimeters O_2 consumed per hour per milligram dry weight of tissue.

RESULTS

The results for the Q_{O_2} values of testes of the six groups of rats are summarized in the fourth column of Table 1. Comparisons of the percentage differences in Q_{O_2} values for testes from normal rats and from each of the five remaining groups follow: adrenalectomized (+72 per cent), "light" (+139 per cent), and "heavy" (+50 per cent) dosages of testosterone propionate, and adrenalectomized rats previously treated with "light" (+17 per cent) and "heavy" (+22 per cent) dosages of testosterone propionate. This table, which is self-explanatory, gives the various data pertinent to a statistical comparison of the difference between any particular pair of mean Q_{O_2} values. Comparisons of the differences between the indicated pairs of mean Q_{O_2} values (last column in table) show that all combinations of data are significant except those from either group of testosterone-treated adrenalectomized rats and normal rats; from rats treated with "heavy" dosage of testosterone propionate and adrenalectomized rats; and from the two groups of adrenalectomized rats which were previously given prolonged treatments of "light" and "heavy" dosages of testosterone propionate, respectively.

DISCUSSION

The results show a significant increase (72 per cent) in the mean Q_{O_2} values of testes from adrenalectomized as compared with those from normal rats. These results are contrary to those reported by Himwich, Fazekas, Barker, and Hurlburt (1934), who found a 43 per cent decrease in oxygen consumption of seminiferous tubules from adrenalectomized rats. The discrepancy between these results and those of Himwich *et al.* may be due to (*a*) the difference in method of standardizing the data, (*b*) the method of sampling the tissue, or (*c*) the time elapsing between adrenalectomy and testectomy. In answer to the questions raised, (*a*) although Himwich *et al.* employed "the technique developed by Warburg," it is evident from their data that standardization of their values for oxygen consumption is based on wet, instead of on dry, weights. Since water tends to shift into certain tissues and cells following adrenalectomy (Gonzalez and Angerer, 1947), an analysis was made based on ten right testes from adrenalectomized and normal rats, respectively. This failed to show a significant change in the water content of this organ. (*b*) Himwich *et al.* made their studies on isolated intact seminiferous tubules,

whereas the present results are based on whole testes. Qualitatively, though not quantitatively, there is no reason to anticipate contrary results between minced and whole or sliced tissue. Exploratory experiments in our hands gave more constant Q_{O_2} values for a given testis when samples were taken from mince than from intact seminiferous tubules. (*c*) Unthe Q_{O_2} is lower than that of the controls.

That testicular Q_{O_2} values following adrenalectomy should show an increase is unexpected. As a result of the studies on total metabolism of adrenalectomized rabbits (Marine and Baumann, 1922) and cats (Aub, Forman, and Bright, 1922) the expected change for the Q_{O_2} of

TABLE 1

SUMMARY AND ANALYSES OF THE MEAN Q_{O_2} VALUES OF TESTES FROM NORMAL AND ADRENALECTOMIZED RATS TREATED FOR LONG PERIODS WITH EITHER "LIGHT" (L.) OR "HEAVY" (H.) DOSAGES OF TESTOSTERONE PROPIONATE (T.)*

Rat 80–120 Gm., Starved 18–21 Hr.	Expt. Groups	No. of Rats and Expts.	Q_{O_2} $M \pm$ S.E. (σ)	*P*-Values for Expt. Groups Indicated
Normal (N.) (controls)	A	16	-1.8 ± 0.18 (0.73)	B<0.01, C<0.01, D<0.01, E>0.05, F>0.05
Adrenalectomized (A.)	B	25	-3.1 ± 0.21 (1.07)	C<0.01, D>0.05, E<0.01, F<0.01
L.T.+N	C	18	-4.3 ± 0.26 (0.12)	D<0.01, E<0.01, F<0.01
H.T.+N	D	18	-2.7 ± 0.11 (0.11)	E<0.01, F<0.01
L.T.+A	E	20	-2.1 ± 0.16 (0.72)	F>0.5
H.T.+A	F	19	-2.2 ± 0.12 (0.53)	.....

* Q_{O_2} column contains: mean value (M) $\pm$ standard error (S.E.) and standard deviation (σ) in parentheses. The last column gives the respective *P*-values derived from a comparison between a given pair of mean Q_{O_2} values, as indicated by the specific letter which corresponds to a given rat group (2d col.) and the rat group as identified by the line indicating a specific rat group (1st col.) on which the letter in question is found.

doubtedly the crux of the apparent discrepancy lies here. Himwich *et al.* obtained tissue from adrenalectomized rats *in extremis*, whereas our tissue was from adrenalectomized rats which appeared normal in behavior. In the light of the cardiovascular collapse (Swingle, Parkins, Taylor, and Hays, 1936) accompanying adrenocortical insufficiency, it is suggested that the resulting stagnant anoxia and moribund condition may alter the results. It may be that the Q_{O_2} of testis is higher during the early period of adrenocortical insufficiency, while *in extremis* the constituent tissues is a decrease (kidney—Tipton, 1941, and Russell and Wilhelmi, 1941; liver—Tipton, 1941; erythrocytes—Gonzalez and Angerer, 1947; aorta—Angerer, 1946, and Arscott and Angerer, 1949). Butcher (1943), however, though interested in hair growth following adrenalectomy, reported an increase in postoperative Q_{O_2} values of the isolated skin of rat. He attributed this increased metabolism to the hair follicle. Since this time, two other tissues have been found to give an increase in Q_{O_2} values (heart—Angerer and Gon-

zalez, 1947; testis—Gonzalez and Angerer, 1947).

The Q_{O_2} of testes from normal rats treated with "light" and "heavy" dosages of testosterone propionate showed an increase of 139 and 50 per cent, respectively, when compared with controls (normals). These results may be interpreted on the basis of the work of Moore and Price (1930, 1932), which treats of the reciprocal relation between testosterone and gonadotrophin secretions. An inhibition of the secretion of gonadotrophin suggests a reduction in testicular metabolism. This, however, is contrary to the observed results. Previously, Angerer and Gonzalez (1950) showed that "light" dosages of testosterone propionate produced a decrease in the water content of these organs without a decrease in protoplasmic solids, whereas "heavy" dosages, while causing a greater decrease in water content, also produced a significant decrease in protoplasmic solids. It suggests that where "light" dosage is used, there is an increase in oxygen consumption due to intracellular reorganization, which is incipient to atrophy. This reorganization is confirmed by a histological study of these testes. "Heavy" dosage of testosterone yields Q_{O_2} values lower than those for "light" dosage, along with atrophy (based on solid-weight change). Had a more heavy dosage of testosterone propionate been employed, it is suggested that the Q_{O_2} values would have been further decreased, possibly to values below the controls.

Since testes from rats treated with either "light" or "heavy" dosages of testosterone propionate or from adrenalectomized rats gave increased Q_{O_2} values compared with their controls, a synergistic response may be anticipated when both procedures are combined in the same rat. The observed testicular Q_{O_2} values for testosterone-treated adrenalectomized rats were no different than for normal rats. It is suggestive to interpret these results as due to the atrophic changes which are already advanced, whereas the results for testes from testosterone-treated rats may be considered as possibly due to the more incipient stages of these changes.

SUMMARY

1. White rats (20–30 gm.) were divided into six groups: (A) normal rats, (B) adrenalectomized rats, two groups of normal rats which received subcutaneous injections of (C) 1 mg. ("light" dosage) or (D) 5 mg. ("heavy" dosage) of testosterone propionate per kilogram of body weight injected 3 ×/week for 5–6 weeks; and two groups (E and F) treated similarly to groups C and D, respectively, but adrenalectomized at least 5 days prior to experimental use. Aliquot samples of minced right testes of five or six rats were studied at one time by the Warburg manometric technique.

2. The Q_{O_2} values ($-mm^3$ O_2/mg dry wt/hr) for the testes of the foregoing six rat groups follow: (A) -1.8 ± 0.18; (B) -3.1 ± 0.21; (C) -4.3 ± 0.26; (D) -2.7 ± 0.11; (E) -2.1 ± 0.16; (F) -2.2 ± 0.12.

3. Comparisons of the six groups of rats on the basis of the difference between any possible combination of a pair of mean Q_{O_2} values are statistically significant ($P < 0.05$) except those from either group of testosterone-treated adrenalectomized rats and the normals (controls); from "heavy" dosage of testosterone-treated rats and adrenalectomized rats; and from the two groups of adrenalectomized rats which had previously been given prolonged treatments of "light" and "heavy" dosages of testosterone propionate.

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OBSERVATIONS ON THE REACTION OF PELOMYXA CAROLINENSIS SUBJECTED TO A DIRECT-CURRENT ELECTRIC FIELD

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NUMEROUS studies have been made on the effect of both alternating and direct current on various Sarcodina. In many instances the effect of electrolytic products formed at the electrodes could not be evaluated, and highly reproducible current densities could not be obtained. The present report describes some of the reactions of *Pelomyxa* in a system designed to minimize the foregoing difficulties.

APPARATUS

Figure 1 shows diagrammatically the apparatus used for observing the *Pelomyxa*. The glass observation cell (*F*) is of rectangular cross-section, 12.7 mm. wide. Contact with the source of potential is made through the saline bridges (*C*), magnesium chloride solution (*A*), and magnesium-rod electrodes (*B*) which are insulated to within a few millimeters of their immersed ends. A Cello-

phane seal over the electrode end of each bridge prevents siphoning. The saline in the bridges is the same composition as that used in the observation cell. The total salt-solution path is 22 cm. The auxiliary platinum electrodes (G), having a separation of 1 cm., serve not only to measure the potential gradient but also to obtain reproducible solution depths and to detect the presence of

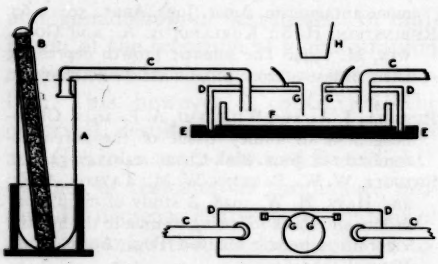

FIG. 1.—Side and top view diagrams of apparatus used to observe reaction of Pelomyxa to electric current.

electrolytic products which may diffuse into the cell from their origin at the Mg-$MgCl_2$ junction.

The direct current through the cell is furnished by the power supply and the vacuum tube T_1 (Fig. 2). With this arrangement the cell current measured by M_1 will depend only on the setting of R_1 and not on the amount or concentration of the solution in the cell. This permits a more rapid and convenient method of obtaining desired values of current density than can be obtained with a battery supply.

The auxiliary electrodes are connected to the vacuum-tube voltmeter and the source of the adjustable bucking voltage V, obtained from battery B_1. With S_1 open and no current through the cell, there will be no voltage drop across the cell, and M_2 will show a constant reading. When voltage is applied to the cell, the voltage drop across the cell will make the grid of T_2 negative, and the reading of M_2 will drop sharply. By adjustment of R_2, M_2 can be returned to its original reading, and the reading of V will then equal the potential difference across the auxiliary electrodes, since the bucking potential will just equal the voltage drop across the cell. Since the current drawn from the cell by the vacuum-tube voltmeter is exceedingly small —less than 10^{-9} amperes—this method does not disturb the current flow through the cell.

The potential difference across the electrodes will be

$$V = \frac{K s I}{b d} \qquad (1)$$

where V = voltage across electrodes, K = constant depending only on the solu-

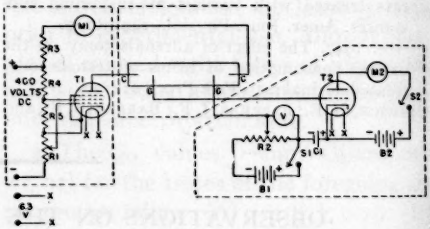

FIG. 2.—Diagram of electrical circuit used in conjunction with the observation cell. Components: $M1$, 0–3 ma.; $R1$, 3,000 ohms, 10 w.; $R3$, 4,000 ohms; $R4$, 500 ohms, 10 w.; $R5$, 25,000 ohms, 10 w.; $T1$, $6SH7$; $R2$, 0.5 megohm; $B1$, 180 v., $C1$, 3 v.; $T2$, $6C5$, $M2$, 0–25 ma.; V, 0–50 v.; $B2$, 135 v. Broken-line blocks: *left*, current control; *center*, observation cell; *right*, vacuum-tube voltmeter.

tion conductivity, I = cell current, s = electrode separation, b = cell width, and d = solution depth.

Electrode separation and cell width are fixed by the construction of the cell, and K will be a constant for a given cell solution. From Equation (1), therefore, V will be proportional to $1/d$ if I is held constant. Solution depths were determined with a micrometer, and the pre-

dicted relationship was verified. If a standard solution is used in the cell, a calibration curve relating V and d can be made for any desired value of I, and then readings of V serve to determine the solution depth and hence the current density. If a current other than the calibration current is used, the d-values determined from the calibration data must be corrected by the ratio of the two currents.

In operation a small amount of saline is placed in the observation cell and R_1 adjusted to obtain the desired value of current as read on M_1. Saline is then added until the desired voltage drop, as measured by the vacuum-tube voltmeter and the bucking voltage, is obtained. This serves to determine the depth of the solution. Because of the action of T_1, the current through the cell will remain constant throughout this adjustment and any subsequent operations. If the level or composition of the solution changes, there will be a corresponding change in the voltmeter reading. With a constant solution level, any appreciable diffusion of electrolytic products through the saline bridges will result in a change in the voltmeter reading. With the arrangement described, no change has been observed in runs lasting more than 1 hour.

In this report current densities are expressed as milliamperes per square millimeter of cross-section of the saline in the observation cell.

With the exception of a few observations on *Amoeba proteus*, the experiments reported in this paper were made on *P. carolinensis* (Wilson) grown in large dishes containing Chalkley's saline (NaCl, 0.1 gm.; KCl, 0.004 gm.; $CaCl_2$, 0.006 gm.; H_2O to 1 liter) to which a few grains of polished rice had been added. Paramecium and a small unidentified flagellate were supplied as food. Cells for the experiments were chosen from cultures 2–3 weeks old. It is an easy matter to select from a vigorous culture of *Pelomyxa* ten or a dozen relatively "large" and a similar number of "small" cells. Such a procedure was used in this study, since at the present time there is, to our knowledge, no satisfactory way to measure the volume of *Pelomyxa*. Chalkley (1929) has described the "volumescope" which he used on *A. proteus*. Belda (1942), employing this method to measure *Pelomyxa*, reported volume changes attributed to water loss. It appears that Belda encountered some disintegration of cells following his experimental manipulation. In our hands the use of the volumescope of a size to give accurate values has resulted in such a high percentage of damaged organisms that we have been disinclined to accept as entirely normal any organism so treated. While we have no measurement, other than breaking time, to show that "large" or "small" groups chosen on succeeding days or from different cultures are the same size, we have had no difficulty, even at widely separated times, in choosing groups of "large" cells which showed no statistical difference in mean breaking time in a given current density; the same is true for the "small" groups.

The selected *Pelomyxa* were placed between the platinum electrodes in the observation cell after the saline depth and current strength had been adjusted and the time between initial passage of the current and undisputed rupture of the cell was measured to the nearest 0.01 minute. The saline added to the observation cell when introducing the *Pelomyxa* was minimal in amount and identical with that in the experimental equipment. The breaking time was determined separately for each cell, and the saline in the observation cell and in the bridges was changed after each measurement.

EXPERIMENTAL RESULTS AND DISCUSSION

One of the reactions observed in *Pelomyxa* subjected to sufficiently strong currents is its orientation followed by disintegration or breaking on the side directed toward the anode. In many instances breaking is preceded by a blister-like formation on this side, followed by rupture and the cytoplasmic outflow. While the formation of a clear blister is common, often there is no indication of a clear space between the outer membrane and the granular mass but, rather, a direct impingement of the granules on the membrane prior to its rupture.

In many instances, occurring in either the presence or the absence of blister formation, there is a "leakage" of granules through the membrane without any visible rupture, an observation which may have been limited by the low magnification used. It seems evident, however, that the membranes of different cells rupture in a somewhat altered time sequence. One is inclined to picture a series of weak spots giving way in some cells with appreciable time lapses between ruptures, in others with a rapidity of sequence approaching simultaneity.

Data from a single series of experiments representative of a large number of breaking-time determinations made at intervals over a period of 2 years are given in Table 1. In all the experiments, without exception, the same relationships (not specific averages) have been observed with no overlapping of breaking time of large and small groups. The most striking result is the great difference in breaking time between large and small cells in the same saline and at the same current density.

To study further the relation of size to breaking time, a group of large Pelomyxae was selected from a clone culture grown in Chalkley's saline. Each organism was cut into two pieces of unequal size, which were subjected simultaneously in the observation cell to a current density of 0.016. The larger pieces always broke in a shorter time than did the small ones. By changing the size relationship between the two pieces, the breaking-time factors could be adjusted from 1 to 10 or more. The results from the cutting experiments and those shown in Table 1 demonstrate that breaking time is partially dependent on the volume of the organism and that calcium, while affecting the observed average for a given cell size group does not alter the

TABLE 1

BREAKING TIMES OF *Pelomyxa carolinensis* SUBJECTED TO SPECIFIED CURRENT DENSITIES AND SALINE SOLUTIONS IN AN OBSERVATION CELL 12.7 MM. WIDE

Size Group	No. Used	Saline	Voltage	Total Current (Ma)	Solution Depth (Mm.)	Current Density (Ma/Mm²)	Range of Breaking Time (Min.)	Mean Breaking Time (Min.)
Large.....	26	Chalkley's	5	0.5	2.39	0.016	0.33– 5.23	1.94
Small.....	25	Chalkley's	5	0.5	2.39	0.016	6.76–15.0*	13.06*
Large.....	15	Triple calcium	5	0.5	2.21	0.018	1.00– 5.02	3.32
Small.....	10	Triple calcium	5	0.5	2.21	0.018	15.0†	15.00†
Small.....	10	Chalkley's	12	0.6	1.19	0.040	0.25– 3.75	1.88
Small.....	15	Triple calcium	12	0.6	0.91	0.052	0.20– 3.60	1.86

* The maximum breaking time was not determined. If the organism had not broken within 15 minutes, the experiment was terminated at this point, and 15 minutes was arbitrarily used as the breaking time in calculating the mean breaking time.

† None of the organisms had broken within the 15-minute interval which was arbitrarily selected as the end-point of the experiments.

unique relationship between size and average breaking time.

It is also evident from Table 1 that higher calcium levels increased the current density required to break cells in a specified time. In a cell breaking time of 1.88 and 1.86 minutes, respectively, the current density was increased from 0.040 in Chalkley's saline to 0.052 in triple calcium saline.

In all the experiments it was noted that the degree of orientation of *Pelomyxa* depended upon the strength of the current. This is in agreement with the results of numerous investigators using amoebae (Luce, 1926; Mast, 1941; Hahnert, 1932). In a low current density *Pelomyxa* moved at random in the observation cell, but, with increased current, movement was toward the cathode either by direct reversal of streaming accompanied by pseudopodia formation on the cathodal side, by a gradual turning of the pseudopodia, or by a combination of the two. If the polarity of the electrodes was reversed, the organism also reversed its direction and continued toward the new cathode.

When breaking, *Pelomyxa* always showed rupture on the anodal surface, with direct correlation between the magnitude of the break and the strength of the current. In strong currents the cytoplasmic mass expelled from the organism moved slowly toward the anode for a few moments, subsequently dispersing, with some of the granules migrating toward the cathode while others continued toward the anode. The majority, however, settled rapidly and adhered to the bottom of the observation cell. With the low magnification (×54) used, it was impossible to determine any morphological differences in the granules associated with their direction of movement.

Mast (1941), working with *A. proteus*, observed no migration of granules toward the cathode; all moved toward the anode or settled to the bottom of the observation cell. These findings were confirmed in the present investigation. With reversal of current, the granules of both *Pelomyxa* and *Amoeba* reversed their direction of movement. No clumping of granules could be observed at either pole in the intact *Pelomyxa* or *Amoeba*.

Based on the movement of intact organisms or the granules released on rupture, the general assumption has been that migration is due to the charge on the migrating particle. Observations of others as well as our own provide no sound basis for any conclusions relative to the charge on the cell or on the granule. In many cases the apparatus used to observe the behavior of the intact cell or the expelled granules has been of such nature that a distinction could not be made between the movement due to charge and that resulting from the electro-osmotic flow of water. The intact free-floating *Pelomyxa* or *Amoeba* is near the bottom of the observation vessel, and this is the position in which one would expect to find marked cathodal endosmotic streaming. It has been observed that, with cell rupture and granule dispersion, the upper and lower layers are moving in opposite directions. In a satisfactory electrophoresis cell the water streams toward the cathode along the walls of the vessel, and the return stream is through the center. If the mass of granules tends to spread upward from a point near the bottom of the observation vessel, it is quite likely that some of the granules will be caught in the return stream and apparently be carried under the influence of the electric current, when in reality they are being carried by the water stream. In order to secure correct results, it is necessary to make observations and measurements at the stationary level in the obesrvation cell or to

correct for the flow of water at other levels. Movement toward one pole or the other in an electric field is a necessary, but not a sufficient, criterion of the presence of charge on a particle free to move in relation to the walls of the containing vessel.

SUMMARY

1. An apparatus is described which has certain advantages over the usual equipment employed to study the reaction of protozoan cells to electric currents.

2. The reactions of *P. carolinensis* to a direct current are described, and the current densities required to produce the observed effects are given.

3. At a specified current density, the time required for *Pelomyxa* to break is directly correlated with the size of the cell.

4. At a fixed current density, calcium increases the breaking time of both large and small cells without altering the size–breaking-time relationship.

5. It is pointed out that conclusions concerning the charge on the intact cell or on the granules released at rupture cannot be drawn from the data of this paper or from numerous other reports in the literature.

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THE MODIFICATION OF DEVELOPMENTAL PATTERNS IN THE SAND DOLLAR WITH SODIUM AZIDE[1]

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MANY agents have been used to modify development in the echinoderm egg. Most of these act as inhibitors of developmental processes and tend to behave differentially and in a quantitative manner. The general effects of entirely different agents may be quite similar, but there are many differences. Sodium thiocyanate in Ca-free sea water produces ectodermization if used before fertilization, while lithium chloride under the same conditions causes entodermization (Lindahl, 1933, 1936; Rulon, 1941, 1946). Pease (1941, 1942*a*, *b*) showed that certain agents (sodium azide and other enzyme inhibitors) in steep concentration gradients change the ventrodorsal axis in developing sand-dollar eggs, while other agents (also enzyme inhibitors), although differentially inhibiting cleavage, fail to alter ventrodorsality. Rulon (1949) found that maleic acid (an inhibitor of succinic dehydrogenase and glyoxalase) produces a high percentage of differentiated radial plutei

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when applied at the blastula stage. This type of modification is usually not found with other inhibitory substances. Numerous agents (see Child, 1948, for review) readily cause exogastrulation and entodermization in echinoderm eggs, while a number of experiments with sand-dollar material have shown maleic acid to be relatively ineffective in producing modifications of this kind. There are other differences.

The present work was undertaken to test the effects of sodium azide on sand-dollar development. This agent was chosen because recent work has shown it to be a particularly good inhibitor of certain important biological processes.[2]

MATERIAL AND METHODS

The experiments were performed only on the eggs of the sand dollar, *Dendraster excentricus*. The adults were dredged in large numbers from Monterey Bay during July, 1948, and were maintained in aquaria of running sea water at the Hopkins Marine Station. After being washed in tap water, the animals were opened with scissors, and only eggs exuding from ripe red ovaries were used. These were drawn up into a medicine dropper in thick masses and washed several times with sea water before being fertilized with a few drops of sperm suspension. Fertilization was usually 95–100 per cent.

Fresh solutions of M/100 sodium azide in sea water were made up and diluted to the concentrations used in the experiments. The developing eggs were kept in covered finger bowls out of direct sunlight at one end of the aquarium room, at a temperature that varied by no more than 1° from 18° C. Everyeffort was made to prevent crowding of eggs in both test and control lots. Smoking was not permitted in the room during the course of the experiments.

Many preliminary experiments were run with concentrations of azide ranging from M/100 to M/4,000. In the final experiments, described in this paper, only the most effective range (M/200–M/800) was used, although additional interesting data may be obtained by extending the range, using different concentrations within the range, different exposure periods, and different stages of exposure. In so far as the objectives of this work are concerned, it is believed that they have been accomplished, even though the experiments here described are far from exhaustive.

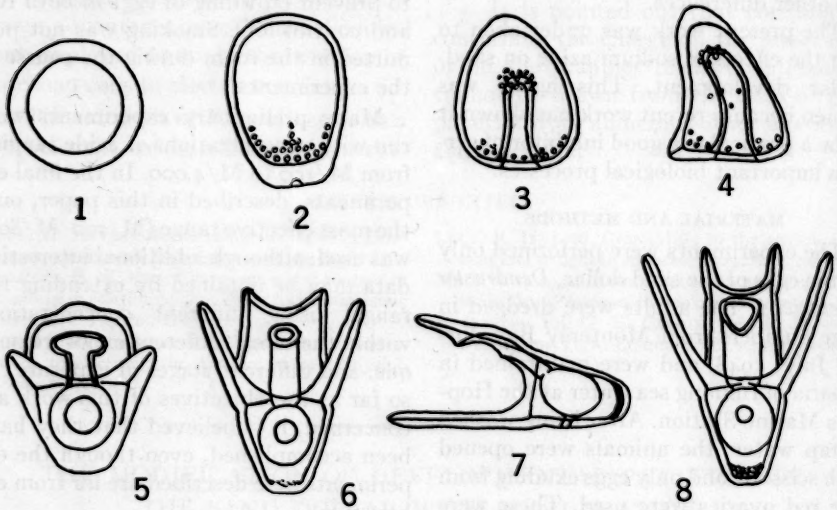
NORMAL DEVELOPMENT

Since the normal development of the egg of *Dendraster* has been previously described (Child, 1940; Rulon, 1941, 1949), only brief attention will be given it here.

By 6 hours after fertilization at a temperature of 18° C., the smooth, thick-walled, blastula stage is reached (Fig. 1). Ciliary movement has not yet begun, and the fertilization membrane is still intact. By 12 hours the larva has become free-swimming and elongated in the polar axis (Fig. 2). The walls have become thin except in the vegetal region, primary mesenchyme has appeared, and there may be a trace of invagination. By 18

[2] Sodium azide is known to inhibit cytochrome oxidase, catalase, and other iron porphyrin enzymes (University of Wisconsin Biochemists, 1939, p. 39; Green, 1941, pp. 19, 32, etc.; Singer, 1945). It inhibits certain synthetic processes which are essentially anaerobic (see Spiegelman and Moog, 1945, for references) and protein synthesis (Melchior, Mellody, and Klotz, 1948). Barth and Jaeger (1947) found that sodium azide inhibition of gastrulation in amphibians was accompanied by increased inorganic phosphate at the expense of ATP content of the gastrula. Hall and Moog (1948) state that certain inhibition of development in amphibians with azide "may be tentatively explained if it is assumed that azide prevents phosphorylations which ordinarily supply energy for developmental processes. . . ." Loomis and Lipmann (1949) have shown inhibition of phosphorylation by azide in kidney homogenates. There are many other references on the effects of azide on vital processes.

hours the gut has invaginated, ventrodorsality has developed, and the apical end has become secondarily thickened (Fig. 3). At 24 hours the ventral surface is flattened or slightly concave, the gut is directed ventrally, and the anal arms are making their first appearance in basal-lateral positions of the ventral area (Fig. 4). By 30 hours the ventral surface is more concave, coelomic pouches are present, the oral lobe is taking shape, and the anal arms are longer and with good skeletal development (Fig. 5). At 42 hours the anal arms are longer, the oral lobe narrower, and the oral arms are appearing (Fig. 6). The 60-hour pluteus (Figs. 7, 8) is well developed, with long oral and anal arms. Under laboratory conditions and without feeding, the larvae usually do not go far beyond this stage.



FIGS. 1–8.—Normal development of the sand dollar from the 6-hour blastula to the 60-hour pluteus

EXPERIMENTAL

1. CONTINUOUS EXPOSURE TO SODIUM AZIDE FOLLOWING FERTILIZATION

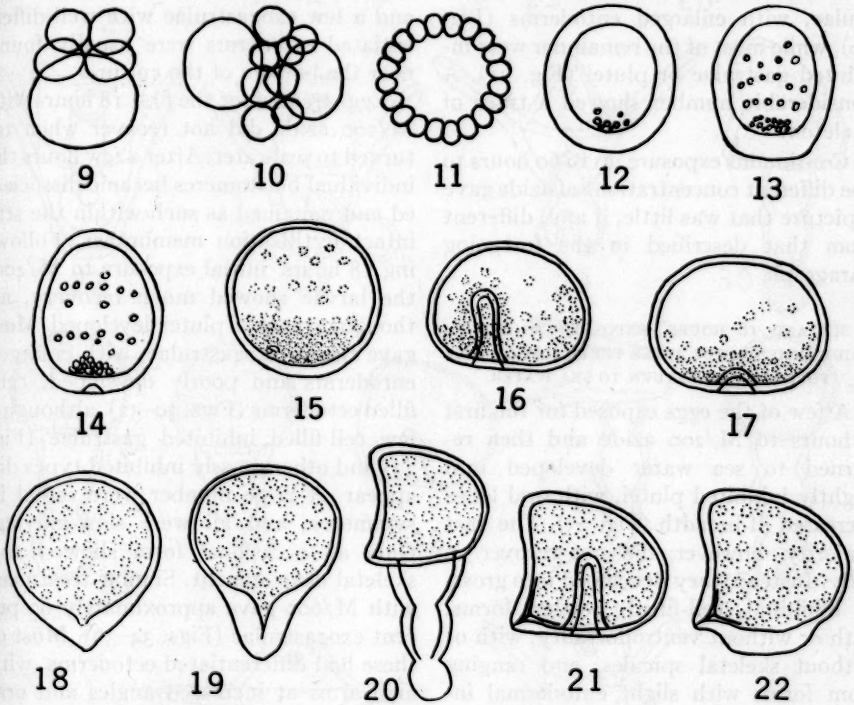
Newly fertilized eggs, showing 95–100 per cent fertilization membranes, were placed in M/200, M/400, M/600, and M/800 sea-water solutions of azide and, along with sea-water controls, were observed at definite intervals.

After 6 hours, practically all the eggs in the sea-water control were in the normal mid-blastula stage (Fig. 1), while eggs exposed to M/200 azide were in 2–16-cell stages, with blastomeres usually of unequal size and cleavage pattern irregular (Figs. 9, 10). Exposure to M/400 azide for this length of time gave forms ranging from late cleavage to early blastulae, with large, easily discernible cells (Fig. 11). Those in M/600 were slightly more advanced, but the cells were still large, and development was still behind the control. In M/800 the eggs had advanced to the mid-blastula stage, with cells only slightly larger than in the control.

After 18 hours, when the controls had reached the free-swimming mid-gastrula stage (Fig. 3), the eggs in M/200 azide were still in early irregular cleavage, with the large blastomeres often multinucle-

ate. Those in M/400 were still at the bottom of the culture but were exhibiting slow movement. For the most part they were thick-walled radial blastulae with a trace of mesenchyme (Fig. 12). A few, however, showed an excess of internal cells. At this time the eggs in M/600 had developed chiefly into slow-moving, bottom, radial forms, commonly with an excess of mesenchyme. Several seemed to be near the beginning of gastrulation (Fig. 13). In M/800 the eggs, for the most part, had reached the late blastula to early gastrula stages and were usually free-swimming. Most were radial in symmetry, but some were beginning to develop bilaterality (Fig. 14).

After 42 hours in M/200 azide the eggs had become dissociated completely, and the individual cells were held together only by the still intact fertilization membranes. After 46 hours in M/400 all were bottom, slow-moving, thin-walled, spherical forms (Fig. 15). Most were slightly thicker at the vegetal pole and contained an excess of internal cells. There was little or no evidence of gastrulation or ventrodorsality. Many were small in size, a condition that usually indicates loss of cells or fission during development. After 47 hours in M/600 the larvae were, for the most part, radial bottom forms, with fairly slow movement, an excess of internal cells, and ranging from forms with considerable invagination of gut to varying degrees of exogastrulation (Figs. 16–



FIGS. 9–22.—Sand-dollar larvae developing in various concentrations of sodium azide, with exposure beginning at the 1-cell stage.

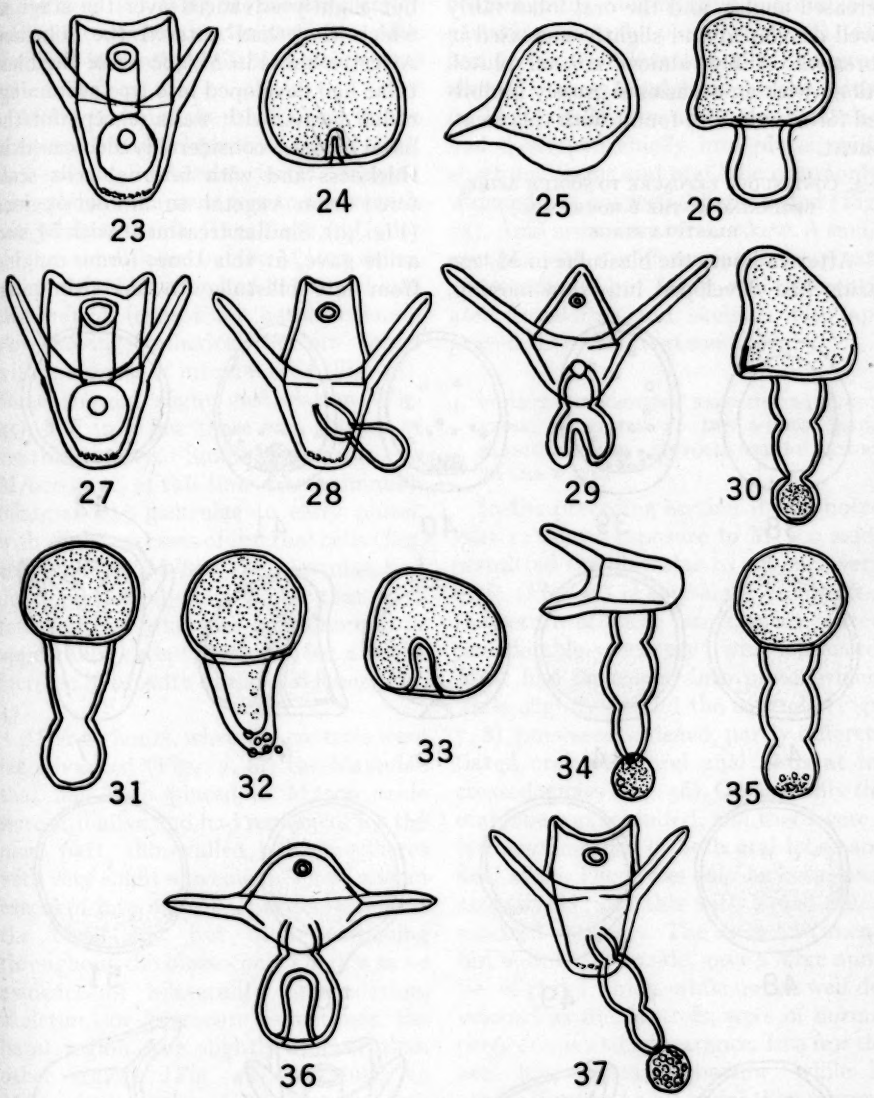
19). There was very little ectodermal differentiation, although in a few there was a trace of ventrodorsality. After 47 hours in M/800 azide the larvae were almost all at the bottom of the culture, although exhibiting considerable movement. All had an excess of internal cells, and practically all showed ventrodorsality (Figs. 20–22). Over 50 per cent were exogastrulae, with enlarged entoderms (Fig. 20), while most of the remainder were inhibited gastrulae or plutei (Fig. 21). A considerable number showed a trace of skeleton.

Continuous exposure up to 60 hours to the different concentrations of azide gave a picture that was little, if any, different from that described in the foregoing paragraph.

2. SIX AND 18 HOURS' EXPOSURE TO SODIUM AZIDE IMMEDIATELY AFTER FERTILIZATION AND FOLLOWED BY RETURN TO SEA WATER

A few of the eggs exposed for the first 6 hours to M/200 azide and then returned to sea water developed into slightly inhibited plutei, with oral lobes increased in breadth (Fig. 23). The vast majority, however, did not recover so fully. Instead, they developed into grossly inhibited, cell-filled, bottom forms, with or without ventrodorsality, with or without skeletal spicules, and ranging from forms with slight entodermal invagination (Fig. 24) to forms with a certain degree (usually not extensive) of entodermal evagination (Figs. 25, 26). Similar treatment with M/400 azide gave, for the most part, fairly normal plutei which were only slightly behind the controls. In a few the oral lobe was wider than normal (Fig. 27), while in others it was definitely decreased in breadth (Figs. 28, 29), sometimes to such an extent that the skeletal spicules came together at a point (Fig. 29). It was also interesting to note that those larvae with narrow (or inhibited) oral lobes commonly showed anal arms of considerable length and at increased angles. Also, in many the gut had evaginated, although showing differentiation. Six-hour treatment with M/600 and M/800 usually gave plutei that were only slightly behind the controls. However, increased angles to anal arms were not uncommon, and a few exogastrulae with well-differentiated ectoderms were usually found near the bottom of the culture.

Eggs treated for the first 18 hours with M/200 azide did not recover when returned to sea water. After a few hours the individual blastomeres became dissociated and remained as such within the still intact fertilization membranes. Following 18 hours' initial exposure to M/400, the larvae showed much recovery, although no normal plutei developed. Most gave rise to exogastrulae, with enlarged entoderms and poorly developed, cell-filled ectoderms (Figs. 30–32), although a few cell-filled inhibited gastrulae (Fig. 33) and other grossly inhibited types did appear. A large number were radial in symmetry, and all were slow-moving. Only an occasional form showed any skeletal development. Similar treatment with M/600 gave approximately 90 per cent exogastrulae (Figs. 34–36). Most of these had differentiated ectoderms, with anal arms at increased angles and oral lobes commonly (but not always) inhibited (Fig. 36). Usually the entoderm was increased far above the normal, while occasionally the ectoderm remained radial and showed little differentiation (Fig. 35). Cell-filled gastrulae and other grossly inhibited forms were also sometimes found in the culture. Eighteen hours' exposure to M/800 also gave approximately 90 per cent exogastrulae (Fig. 37), but in these the ectoderm was larger (and better differentiated) and the entoderm smaller than in those treated with M/600

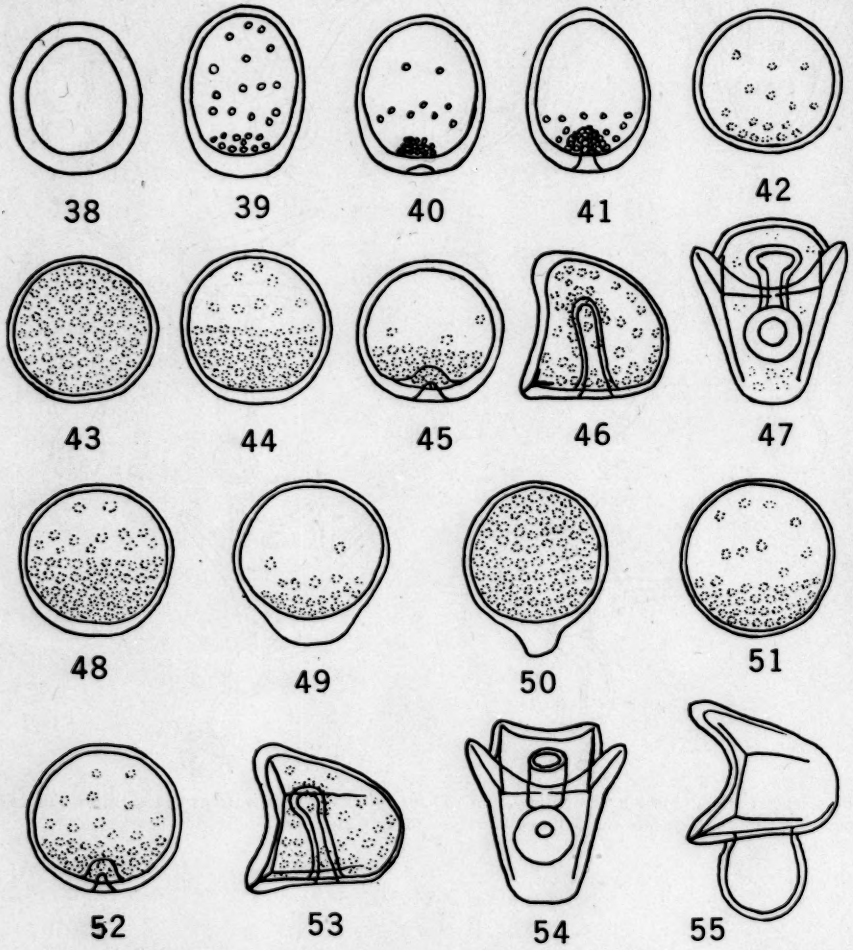


FIGS. 23–37.—Sand-dollar larvae after 6 and 18 hours of exposure to various concentrations of azide immediately after fertilization and followed by return to sea water.

azide. The anal arms were usually at increased angles, and the oral lobes fairly well developed and slightly increased in breadth. A few almost normal plutei, along with an occasional grossly inhibited form, were also found with this treatment.

3. CONTINUOUS EXPOSURE TO SODIUM AZIDE, BEGINNING AT THE 6-HOUR (MID-) BLASTULA STAGE

After 12 hours the blastulae in M/200 azide had developed into slow-moving, thick-walled forms (Fig. 38) that were but slightly advanced over the stage at which they had entered the solution. After 12 hours in M/400 azide the blastulae had developed into free-swimming, radial forms, with walls (except for the basal region) considerably decreased in thickness and with internal cells scattered from vegetal to animal regions (Fig. 39). Similar treatment with M/600 azide gave, at this time, forms ranging from late blastulae to free-swimming,



FIGS. 38–55.—Sand-dollar larvae developing in various concentrations of sodium azide, with exposure beginning at the 6-hour blastula stage.

radial, and somewhat stunted, early gastrulae (Fig. 40). With M/800 azide under similar conditions early gastrulae with the first indications of bilaterality had appeared (Fig. 41).

After 36 hours in M/200 azide the blastulae had developed into thin-walled, slow-moving, spherical forms, ranging from those with very few to those with many internal cells (Figs. 42–43). Under similar conditions, but in M/400 azide, most of the blastulae had developed into thin-walled (except for basal regions), slow-moving, spherical bottom forms with an excess of internal cells (Fig. 44). Some showed slight gastrulation (Fig. 45), and in a few there was a trace of ventrodorsality. Similar exposure to M/600 gave, at this time, free-swimming bilateral late gastrulae to early plutei, with slight excesses of internal cells (Fig. 46), while in M/800 the blastulae had developed chiefly into plutei that were retarded but otherwise fairly normal in appearance except, perhaps, for a slight increase in breadth of the oral lobes (Fig. 47).

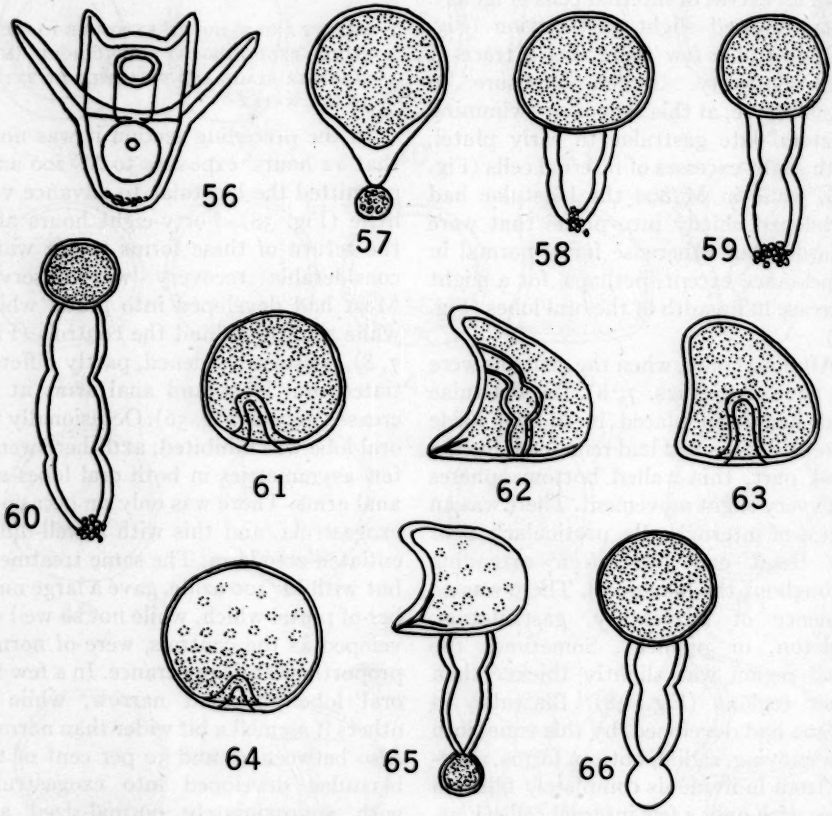
After 60 hours, when the controls were far advanced (Figs. 7, 8), the blastulae that had been placed in M/200 azide were still alive and had remained, for the most part, thin-walled bottom spheres with very slight movement. There was an excess of internal cells, particularly near the basal end but often extending throughout the blastocoel. There was no evidence of bilaterality, gastrulation, skeleton, or pigment. Sometimes the basal region was slightly thicker than other regions (Fig. 48). Blastulae in M/400 had developed, by this time, into slow-moving, radial, bottom forms, ranging from individuals completely filled to those with only a few internal cells (Figs. 49–52). Commonly the basal region was considerably thickened and protruding (Figs. 49, 50). Many also showed slight gastrulation (Fig. 52). Similar treatment with M/600 azide gave all slow-moving bottom forms, ranging from bilateral late gastrulae to early plutei (Fig. 53). All had an excess of internal cells, and most showed some skeletal development. After 60 hours in M/800 azide the blastulae had developed chiefly into plutei, with short anal arms and oral lobe commonly widened but not well differentiated (Fig. 54). Anal arms were often askew. A small number with undifferentiated, evaginated entoderms but fairly well-differentiated ectoderms and skeleton also appeared with this treatment (Fig. 55).

4. TWELVE AND 36 HOURS' EXPOSURE TO SODIUM AZIDE, BEGINNING AT THE 6-HOUR (MID-) BLASTULA STAGE AND FOLLOWED BY RETURN TO SEA WATER

In the preceding section it was noted that 12 hours' exposure to M/200 azide permitted the blastulae to advance very little (Fig. 38). Forty-eight hours after the return of these forms to sea water, considerable recovery was observed. Most had developed into plutei which, while slightly behind the controls (Figs. 7, 8), possessed widened, partly differentiated oral lobes and anal arms at increased angles (Fig. 56). Occasionally the oral lobe was inhibited, and there were a few asymmetries in both oral lobes and anal arms. There was only an occasional exogastrula, and this with a well-differentiated ectoderm. The same treatment, but with M/400 azide, gave a large number of plutei which, while not so well developed as the controls, were of normal proportions and appearance. In a few the oral lobe appeared narrow, while in others it seemed a bit wider than normal. Also between 40 and 50 per cent of the blastulae developed into exogastrulae with approximately normal-sized and well-differentiated ectoderms and with differentiated, almost normal-sized (though evaginated) entoderms. The picture was not far different when the blas-

tulae were treated with M/600 azide and then returned to sea water. Many normal plutei developed, but here again approximately 50 per cent of the blastulae gave rise to exogastrulae with well-differentiated ectoderms and evaginated, differentiated, normal-sized entoderms. Under similar conditions, but with M/800 azide, mostly free-swimming, well-developed plutei appeared. Only a few exogastrulae developed, and those with normal-sized and differentiated ectoderms and entoderms.

When blastulae were returned to sea water after 36 hours' exposure to M/200 azide, they had developed chiefly into thin-walled spherical forms containing from a few to many internal cells (Figs. 42, 43). After 24 hours in sea water these forms still had polarity, but most had lost the capacity for developing ventrodorsality. A large majority were radial exogastrulae (Figs. 57–60) with cell-filled, thin-walled, undifferentiated ectoderms, usually considerably reduced in size, and with greatly enlarged entoderms (Figs. 59, 60). There was a small number of free-swimming, cell-filled gas-



FIGS. 56–66.—Sand-dollar larvae showing effects of 12 and 36 hours' exposure to various concentrations of azide, beginning at the 6-hour blastula stage and followed by return to sea water. Fig. 56, differentially recovered pluteus following 12 hours' exposure to M/200 azide. Figs. 57–66, larvae developing after 36 hours' exposure to M/200 and M/400 azide.

trulae with small, undifferentiated invaginations, sometimes directed toward one side (presumably the ventral), which gave the only indication of a ventrodorsal pattern (Fig. 61). No ectodermal differentiation was noted, although occasionally a skeletal spicule was seen. Comparable treatment with M/400 azide caused many of the blastulae to develop into free-swimming, inhibited, cell-filled forms (Figs. 62–64), ranging from early plutei with short anal arms, oral lobe, and differentiated gut (Fig. 62) down to radial gastrulae with but slight entodermal invagination and no differentiation of ectoderm (Fig. 64). More than half the forms resulting from this treatment, however, developed into exogastrulae with enlarged entoderms and with ectoderms grading from those that were bilateral and showed some differentiation (Fig. 65) to those that were completely radial, cell-filled, and with no differentiation (Fig. 66).

DISCUSSION

This work with azide has shown little or nothing as regards *specific* effects on developmental patterns in *Dendraster*. Practically all (and many more) of the modifications caused by azide on this form have been produced with lithium (Child, 1940) and other agents (see Child, 1948, for review) and may very well be interpreted on the basis of differential inhibition, conditioning, or recovery in the gradient pattern.

As indicated before (n. 2), azide has a number of known (and undoubtedly many unknown) effects on metabolic processes. Such multiple action may account for the almost complete inhibition of differentiation found on continuous exposure to high concentrations and for the high degree of proportionate recovery following short time exposures. However, with certain high concentrations and long exposure periods, recovery (following azide treatment) is differential (disproportionate). This would seem to indicate that certain activities concerned with differentiation in the developing egg are less affected by azide than are other activities. The fact that azide inhibits early cleavage stages to a greater extent than it does mid-blastula stages is not specific, since maleic acid under similar conditions (Rulon, 1949) shows the mid-blastula to have a greater degree of tolerance.

The high percentage of exogastrulae with inhibited ectoderms and enlarged entoderms is certainly not a specific effect of azide. Numerous and very different agents (lithium, pilocarpine, thiocyanate, cyanide, tobacco smoke, crowding, etc.) produce similar effects. There are other agents, however, that do not readily cause exogastrulation and may have certain effects quite different from those of azide. Maleic acid (Rulon, 1949) is an inhibitory agent of this type. Not only does it serve as a poor agent in the production of exogastrulae, but under certain conditions it causes the development of a high percentage of differentiated radial plutei. Also the development of a highly active ciliated knob at the apical end of the larva is a common occurrence after maleic acid inhibition. Malonic acid also is a poor agent in the production of exogastrulation but is an effective agent in the production of the apical ciliated knobs (Rulon, unpublished).

One explanation for the difference in action of azide, cyanide, etc., on the one hand, and maleic and malonic acids, on the other, may lie in their effects on different enzymes or enzyme systems in the gradient pattern of the egg. In partial support of this view it should be pointed out that, in the developing egg of *Dendraster*, Child (1941) has shown (through the use of the indophenol blue reaction)

apicobasal and ventrodorsal gradients of what appears to be cytochrome oxidase. Also in the late blastula stage he found that a reduction (in decreased oxygen) of the indophenol blue takes place most rapidly at the basal region. Pease (1942*b*) states that bilateral determination in *Dendraster* eggs "appears to be closely associated with the cyanide-sensitive respiratory mechanism which, in turn, passes through a heavy-metal catalyst (a 'cytochrome oxidase')." Among other effects, azide and cyanide are both good inhibitors of cytochrome oxidase. From these observations it seems not unreasonable to assume that azide may act through the differential inhibition of those regions of highest cytochrome oxidase activity. Such action may explain why the invagination reaction is so greatly inhibited, with resulting exogastrulation, under critical conditions. The increase in size of entoderm at the expense of ectoderm following severe azide treatment may be because of a greater inhibition of cytochrome oxidase and perhaps other apical enzyme activity.

The development of large ciliated apical structures following maleic acid but not azide treatment may lie in the fact that maleic acid inhibits certain enzymes (succinic dehydrogenase, glyoxylase) to a greater extent than it does other enzymes (cytochrome oxidase, etc.). Under such conditions the apical region (with greater cytochrome oxidase, etc., activity), being less inhibited, recovers or becomes conditioned more readily and, perhaps with more substrate available, increases in size beyond the normal. Of course, this hypothesis can be only tentative and must remain so until further work, with other and more specific agents, has been completed. The work of Barth and Jaeger (1947) and Hall and Moog (1948) has indicated the importance of phosphorylations in supplying energy for developmental processes in amphibian eggs. In the final analysis it may well be that the inhibition of phosphorylations (or other processes) through the action of azide is of much greater importance than the inhibition of cytochrome oxidase activity in the modification of developmental patterns in echinoderms.

SUMMARY

1. Six-hour blastulae of *Dendraster* were better able to tolerate (or acquire tolerance for) all the concentrations of azide used than were the newly fertilized eggs. On continuous exposure to the highest nonlethal concentrations, both stages developed into thin-walled, cell-filled spheres with little evidence of differentiation. With lower concentrations, over 50 per cent of the eggs exposed at the 1-cell stage developed into entodermized exogastrulae.

2. A few nearly fertilized eggs exposed for 6 hours to M/200 azide (followed by return to sea water) recovered almost completely, but most developed into cell-filled inhibited forms. As the concentration was decreased, more normal forms appeared, and with M/800 most were fairly normal plutei, although many showed differential recovery of ventral regions.

3. Eighteen hours' exposure to M/200 azide, beginning at the 1-cell stage, was 100 per cent lethal. With the other concentrations (M/400–M/800), approximately 90 per cent of the eggs developed into entodermized exogastrulae when returned to sea water. Ectoderm remained undifferentiated when M/400 was used but recovered differentially when M/800 was used.

4. Six-hour blastulae, exposed for 12 hours to M/200 azide, developed into plutei with increased ventral regions (differential recovery) when returned to sea

water. With M/400 and M/600, ectodermal recovery was better, but many had evaginated entoderms. Recovery was almost complete (and proportionate) following treatment with M/800.

5. Six-hour blastulae exposed for 36 hours to M/200 azide (followed by return to sea water) developed chiefly into highly entodermized forms, with little or no ectodermal differentiation. Similar treatment with M/400 azide gave over 50 per cent entodermized exogastrulae, but most showed some recovery of ectodermal differentiation and ventrodorsality.

6. The possibility that azide may cause certain modifications in the developmental pattern through inhibition of cytochrome oxidase is discussed.

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THE MODIFICATION OF DEVELOPMENTAL PATTERNS IN THE SAND DOLLAR BY THIOUREA[1]

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IN WORKING with the effects of thiourea on the sea-urchin egg, Bevelander (1946) noted that concentrations of 1 per cent or greater completely inhibited cleavage, while lower concentrations (0.5 per cent) specifically inhibited gastrulation. Concentrations of 0.1–0.3 per cent produced a retardation in over-all growth rate. He tentatively suggests that the thiourea inhibits enzyme systems (oxidative enzymes other than cytochrome oxidase) necessary for the growth and differentiation of the sea urchin. Bevelander fails to mention (other than that thiourea acts selectively on gastrulation) important phenomena (differential susceptibility, differential recovery, differential tolerance, exogastrulation, ectodermization, entodermization, etc.) often found when echinoderm eggs are treated with various agents. Since knowledge of the enzyme systems involved in the dynamics of development is so important for a better understanding and since much may be gained by studying modifications produced by enzyme inhibitors, it seemed advisable to attack the problem of thiourea inhibition with an egg (sand dollar) that lends itself more readily to experimental modification than does the egg of *Arbacia*.

MATERIAL AND METHODS

All the experiments were conducted in the aquarium room of the Hopkins Marine Station, Pacific Grove, California, during the summer of 1948. This proved to be an ideal place, since the temperature varied by no more than 1° from 18° C. during the course of this work. The adult sand dollars (*Dendraster excentricus*) were dredged in large numbers from Monterey Bay and maintained in running sea water in the laboratory. After being washed in fresh water, the animals were opened, and only the eggs from females with large red ovaries were used. These eggs were drawn up into a pipette in thick droplets as they exuded from the ovaries and were washed several times in sea water. Fertilization was accomplished by adding a few drops of sperm suspension. It was usually 95–100 per cent complete, since these experiments were performed near the middle of the breeding season.

The test agent, as mentioned before, was thiourea, an agent which inhibits the activity of peroxidase[2] (Sumner and Somers, 1947, p. 219) but may not interfere directly with the cytochrome–cytochrome oxidase system (as noted in thyroid tissue by Lerner and Chaikoff, 1945). Also Bevelander (1946) tentatively suggests that thiourea inhibits oxidative enzymes other than cytochrome oxidase in the sea-urchin egg. The agent was made up fresh in isotonic strength (0.54 M), and definite quantities (5–40 cc.) were added to 100-cc. vol-

[1] The writer is most grateful to Dr. L. R. Blinks, director of the Hopkins Marine Station, Pacific Grove, California, for providing the necessary facilities for this work. A grant from the Graduate School of Northwestern University gave material support to this investigation.

[2] Since the presence of peroxidase in *Dendraster* eggs was not determined, it is not definitely known that the inhibitory effects of thiourea on these eggs are actually due to inhibition of peroxidase activity.

umes of sea water.[3] Eggs (fertilized and unfertilized) were then transferred to the test solutions and, along with sea-water controls, were kept in covered finger bowls out of direct sunlight. Observations were made at regular intervals. Smoking was not permitted in the laboratory during the course of the experiments.

EXPERIMENTAL

1. THE EFFECT OF THIOUREA ON DEVELOPMENTAL PATTERN WITH TREATMENT BEFORE FERTILIZATION

Unfertilized eggs (testing 95 per cent fertile) were placed in the following solutions:

a..... Normal sea water (control 1)
b..... Ca-free sea water (control 2)
c..... 100 cc. Ca-free sea water + 10 cc. 0.54 M thiourea
d..... 100 cc. Ca-free sea water + 20 cc. 0.54 M thiourea
e..... 100 cc. Ca-free sea water + 40 cc. 0.54 M thiourea

After 12 hours the eggs were washed several times with sea water, and a few drops of fresh sperm suspension were then added to each culture.

Ninety minutes after fertilization the eggs from solution *a* (sea-water control) showed approximately 25 per cent in the 2-cell stage, with the remainder still uncleaved. Eggs from solutions *b*, *c*, and *d* were approximately 90 per cent in the 2-cell stage,[4] although from solution *d* the two cells of first cleavage were commonly unequal in size. From solution *e* there was no cleavage, and the cortex on many of the eggs was rough and wrinkled.

After 6 hours the eggs from solution *a* showed approximately 40 per cent development, and most were at the normal mid-blastula stage (Fig. 1). Almost all the eggs from solutions *b* and *c* were developing and were also at the mid-blastula stage. From solution *d* a few of the developing forms compared favorably to control 2, but most showed fission, development of the first two blastomeres separately, or general irregularity of cleavage pattern and blastulation (Figs. 2–5). From solution *e* almost all were in irregular cleavage, with little or no definite pattern.

Twenty-four hours after fertilization the eggs from solutions *a*, *b*, and *c* had practically all reached the free-swimming, normal prepluteus stage (Fig. 6). Those from solution *d* showed about 50 per cent to be free-swimming young plutei comparable to the controls. Of the remainder, all had an excess of internal cells, many were irregular in shape, and many were small or half-sized. Almost all these inhibited forms developed bilateral symmetry, although often grossly inhibited and losing cells from basal regions. At this time no normal forms had developed after prefertilization treatment with solution *e*. In this culture many had cytolyzed. The rest were chiefly cell-filled, irregular, partial-sized, bottom forms. Most were losing cells at the basal regions, and many were fused together. Only an occasional form gave any evidence of gastrulation.

By 48 hours the eggs from solutions *a*, *b*, and *c* had developed into normal young plutei (Fig. 7), although there were many uncleaved eggs at the bottom of culture *a*. Only a few of the eggs from solution *d* had developed into normal plutei. Instead, most were cell-filled bottom forms with short anal arms that were sometimes at aberrant angles. In a few the oral lobe was slightly increased in breadth, but in others it was narrow with decreased ven-

[3] Calcium-free sea water was used in the treatment of unfertilized eggs. Its composition was: distilled water, 10, 571 cc.; NaCl, 307 gm.; KCl, 8 gm.; $MgSO_4$, 66 gm.; $NaHCO_3$, 2 gm.

[4] The author (Rulon, 1946, 1948) and others (Schechter, 1937) have shown that the fertilizable life of echinoderm eggs may be extended by keeping them in low-calcium sea water.

tral area. Many were half-sized, most had gastrulated, and most showed good movement. At this time the eggs from solution *e* had all become dissociated (broken up into individual cells; cytolyzed).

It is evident from these experiments that proper treatment of the unfertilized egg with thiourea may produce certain effects on vital processes which, holding over after fertilization, allow cleavage to proceed slowly and irregularly but do not permit differentiation. This is of particular interest, since NaCNS (an agent which produces effects somewhat similar to thiourea in thyroid metabolism) will, under certain conditions, often cause the development of highly ectodermized forms (Rulon, 1941, 1946).

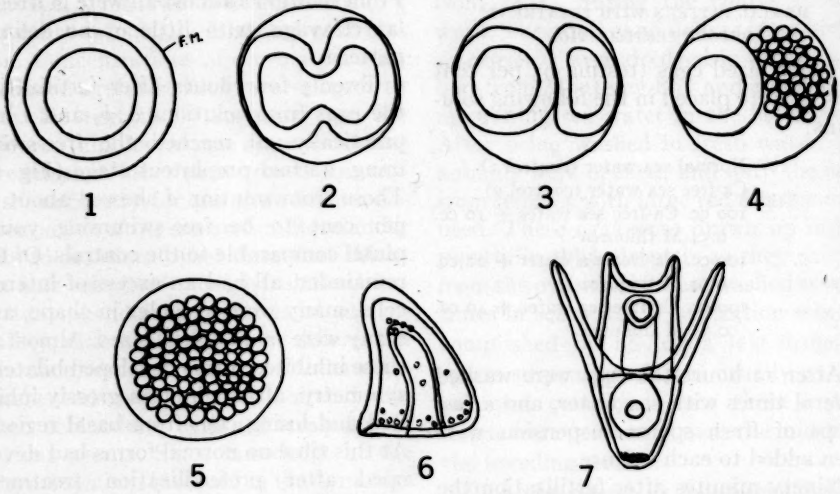
2. CONTINUOUS EXPOSURE TO THIOUREA BEGINNING AT THE 1-CELL STAGE

Newly fertilized eggs were placed in the following solutions.

1 ..... Sea-water control
2 ..... 100 cc. sea water + 5 cc. 0.54 M thiourea
3 ..... 100 cc. sea water + 10 cc. 0.54 M thiourea
4 ..... 100 cc. sea water + 15 cc. 0.54 M thiourea
5 ..... 100 cc. sea water + 20 cc. 0.54 M thiourea

After 6 hours, when the eggs in solution 1 had reached the mid-blastula stage (Fig. 1), the eggs in solution 2 were practically as far advanced but were slightly wrinkled in appearance. With solution 3 the wrinkled condition of the blastula was even more apparent (Fig. 8). Observations of eggs in solution 4 showed that approximately 40 per cent were uncleaved, while the remainder were in irregular and abortive cleavage (Fig. 9). With solution 5, only about 5 per cent cleaved at all, and these cleaved irregularly and abortively (Figs. 10, 11). Eggs in solutions 4 and 5 developed little, if any, further on continuous exposure.

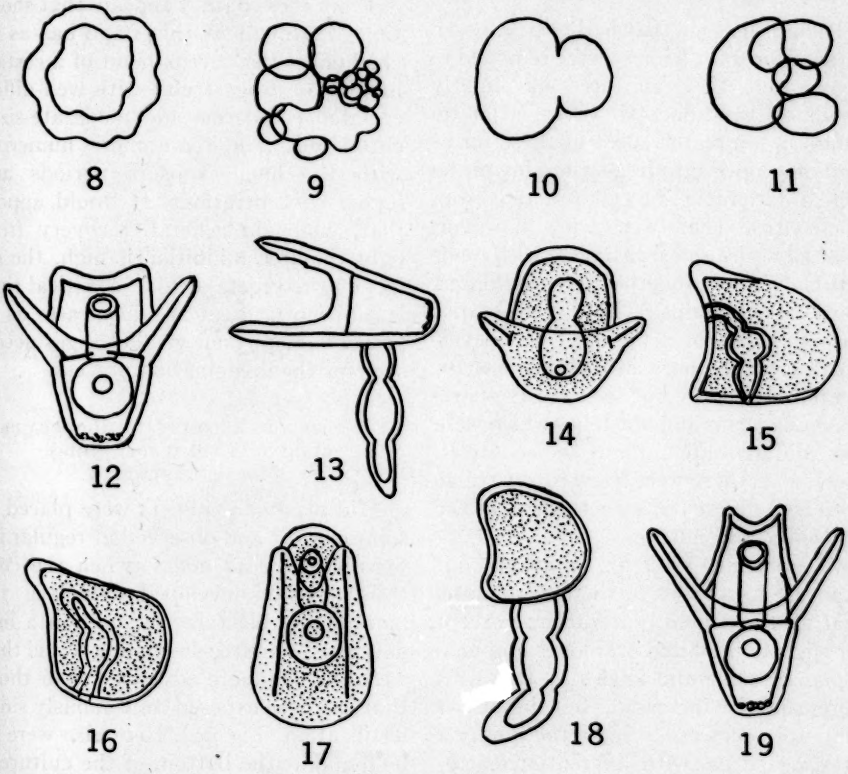
Forty-eight hours' continuous exposure to solution 2 gave free-swimming plutei (Fig. 12) that were definitely less advanced than the control (Fig. 7). The anal arms were shorter, and the oral lobes



FIGS. 1–7.—Fig. 1, normal 6-hour blastula. Figs. 2–5, 6-hour larvae developing in sea water after prefertilization treatment with thiourea. Fig. 6, lateral view of normal 24-hour larva. Fig. 7, anal view of normal 48-hour larva.

were not well differentiated. In some the angles of the anal arms were slightly increased, and an occasional asymmetry was to be noted in both anal arms and oral lobe. On the bottom of the culture was a considerable number (approximately 20 per cent) of exogastrulae with fairly well-differentiated ectoderms and evaginated entoderms only moderately (if at all) increased in size (Fig. 13).

Forty-eight hours' continuous exposure to solution 3 gave a large number of free-swimming, cell-filled forms, with or without short anal arms, with undifferentiated, although sometimes slightly extended and active, oral lobes (Figs. 14–17). Skeletal development was usually slight. In most, the ventral region was only slightly concave (as compared to the control), and, in many, short anal arms were at increased angles (Fig. 14). This condition graded to that in which the ventral region was greatly diminished in size and the angle of the arms so decreased that the skeletal rods were parallel (Fig. 17). The anal region and invaginated gut of most appeared abnormally near the ventral region. Along with many grossly inhibited gastrulae and plutei at the bottom of the culture was found a considerable number (possibly 20 per cent) of entodermized exogastrulae with



FIGS. 8–19.—Figs. 8–11, developing sand-dollar eggs after 6 hours' exposure to increasing concentrations of thiourea. Figs. 12–13, 48-hour larvae that have been exposed continuously since fertilization to weak solutions of thiourea. Figs. 14–18, 48-hour larvae that have been exposed continuously to slightly stronger solutions (sol. 3) of thiourea. Fig. 19, 52-hour larva that was exposed to solution 3 for the first 12 hours and then returned to sea water.

ectoderms poorly differentiated (Fig. 18).

These data show that continuous exposure to thiourea gives a picture of increased inhibition with increased concentrations. Inhibition is differential, as is shown by a decrease in size of apical (the most active of the polar gradient) and ventral (the most active of the ventrodorsal gradient) regions. Entodermization follows the inhibition of basal ectoderm to the physiological level of entoderm.

3. SIX AND TWELVE HOURS' EXPOSURE TO THIOUREA, BEGINNING IMMEDIATELY AFTER FERTILIZATION AND FOLLOWED BY RETURN TO SEA WATER

When the eggs that had been exposed to solution 2 for 6 hours were removed to sea water, they appeared as slightly wrinkled blastulae. However, after 46 hours in sea water, most of these forms had developed into free-swimming plutei which were little, if at all, different from the control. There were a few, however, that gave rise to exogastrulae with moderately sized entoderms and well-differentiated ectoderms. With 6 hours' exposure to solution 3, the eggs had developed, after 48 hours, into plutei that appeared normal except for slightly shortened anal arms and oral lobes which were less differentiated than the controls. Here, also, there were a few exogastrulae with well-differentiated ectoderms at the bottom of the culture.

At 40 hours after an initial 12-hour exposure to solution 2, there were plutei that also appeared quite normal except for slightly inhibited oral lobes and anal arms. In some the angles of the arms were slightly increased, but in others they were decreased. Again there were a few exogastrulae with differentiated ectoderms at the bottom of the culture. With 12 hours' exposure to solution 3 the eggs developed after 40 hours into slightly stunted plutei. In many the anal arms were at slightly increased angles, with the oral lobes slightly more narrow than usual (Fig. 19). In a few cases the oral lobe was slightly increased in breadth and the angles of the anal arms decreased. A sizable number (approximately 20 per cent) of exogastrulae with differentiated ectoderms was also present.

Eggs exposed for 6 and 12 hours to solutions 4 and 5 showed little or no recovery when returned to sea water. Instead, the early-cleavage stages of these eggs gradually became dissociated into component cells and cytolyzed while still within the fertilization membrane.

From these data it appears that short-time treatment at this stage has as its chief effect the development of a certain number of exogastrulae with well-differentiated ectoderms and moderate-sized entoderms. These are more numerous with the long exposure periods and higher concentrations. It would appear that, while, in general, recovery from light thiourea inhibition is high, the effect on the vegetal field at a critical time is sufficiently great in many cases as to prevent the physiological changes necessary for the invagination process.

4. CONTINUOUS EXPOSURE TO THIOUREA BEGINNING AT THE 6-HOUR (MID-) BLASTULA STAGE

Mid-blastulae (Fig. 1) were placed in solutions 1–5 and observed at regular intervals. After 42 hours (when the controls were well-developed plutei [Fig. 7]), most of the blastulae in solution 2 had developed into free-swimming plutei that were slightly more advanced than those that had been exposed continuously since fertilization (Fig. 12). Here also were to be found at the bottom of the culture a considerable number of exogastrulae with differentiated ectoderms and moderate-sized entoderms. After 42 hours in

solution 3 the picture was very similar to that found when the eggs were exposed continuously, beginning at the 1-cell stage (Figs. 14–18).

Although newly fertilized eggs did not long survive continuous exposure to solution 4, this concentration was not lethal for blastulae. After 42 hours they had developed, for the most part, into free-swimming, bilateral, inhibited gastrulae, filled with cells or cellular debris, with no skeletal elements, and with apical (oral) end active and projected ventrally (Fig. 20). Also, a considerable number of entodermized exogastrulae with bilateral, but poorly differentiated, ectoderms appeared at the bottom of the culture (Fig. 21). With solution 5, which permitted only a few abortive cleavages in the newly fertilized egg, mid-blastulae again survived and underwent some differentiation. After 42 hours in the solution, slow-moving forms developed which were filled with cells or cellular debris to such an extent that it was difficult to see inside. Most, however, had some invagination of entoderm, although it had not undergone differentiation (Figs. 22–24). Most gave slight evidence of bilaterality, but even this was lost in some (Fig. 24). It was interesting to note that in those with a definite, though much inhibited, ventral region the invagination of the gut was again nearer this area than in normal development. A few exogastrulae, filled with cells or cellular debris and with grossly inhibited ectoderms and entoderms, were also present (Fig. 25).

These experiments show that continuous exposure of mid-blastulae to solutions 2 and 3 of thiourea gives results not greatly different from those found when the newly fertilized eggs are exposed continuously to the same solutions. This is somewhat surprising, since exposure of these two different stages to the same concentration of certain agents (maleic acid, see Rulon, 1949*a*) often produces quite different effects. However, the blastulae are able to tolerate (or acquire tolerance for) solutions 4 and 5 of thiourea that are lethal to earlier stages and to develop into forms showing extremes of differential inhibition (gross inhibition of the regions showing greatest activity in the untreated larva).

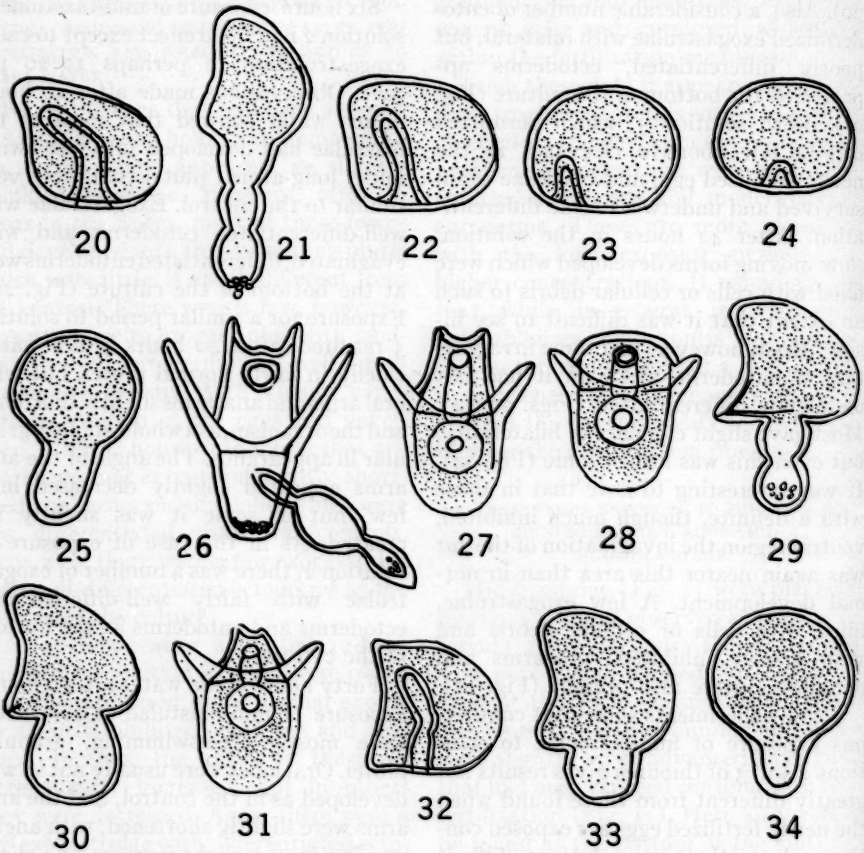
5. SIX AND 18 HOURS' EXPOSURE TO THIOUREA BEGINNING AT THE MID-BLASTULA STAGE AND FOLLOWED BY RETURN TO SEA WATER

Six hours' exposure of mid-blastulae to solution 2 had little effect except to cause exogastrulation in perhaps 10–20 per cent. Observations made after 40 hours in sea water showed that most of the blastulae had developed into free-swimming, long-armed plutei that were very similar to the control. Exogastrulae with well-differentiated ectoderms and with evaginated, differentiated entoderms were at the bottom of the culture (Fig. 26). Exposure for a similar period to solution 3 resulted, after 40 hours in sea water, chiefly in fairly normal plutei, but with oral arms and anal arms slightly shortened and the organism, as a whole, slightly granular in appearance. The angle of the anal arms appeared slightly decreased in a few, but in some it was slightly increased. As in the case of exposure to solution 2, there was a number of exogastrulae with fairly well-differentiated ectoderms and entoderms at the bottom of the culture.

Forty hours in sea water after 6 hours' exposure of mid-blastulae to solution 4 gave mostly free-swimming, granular plutei. Oral lobes were usually not so well developed as in the control, and the anal arms were slightly shortened, with angles varying from a slight increase to a slight decrease. At the bottom of the cultures were still to be found 10–20 per cent exogastrulae with slightly retarded ecto-

derms and moderate-sized entoderms. Similar treatment with solution 5 resulted in definitely inhibited granular, cell-filled forms (Figs. 27–30). Anal arms were short but often at increased angles. Oral lobes were inhibited, and many showed little or no differentiation. In a considerable number the oral lobes had undergone partial differentiation into narrow, but recognizable, structures (Fig. 27). In some a considerable amount of skeleton developed, but in others only a small amount was formed. Entodermal evagination (with little or no entodermization) was seen in many (Figs. 29, 30). In some there were asymmetries of the oral lobes and anal arms.

Eighteen hours' exposure of mid-blastulae to solution 5 gave a picture of gross inhibition. After 30 hours in sea water, there was some recovery compared to those with continuous exposure. All were slow-moving, granular forms which were almost opaque from internal cells and



FIGS. 20–34.—Figs. 20–25, 48-hour larvae exposed continuously to strong solutions of thiourea, beginning at the mid-blastula stage. Fig. 26, exogastrula resulting from 6 hours' exposure to weak solution of thiourea, beginning at mid-blastula stage. Figs. 27–34, larvae resulting from 6–18 hours' exposure to strong solutions of thiourea, beginning at the mid-blastula stage.

cellular debris. There was a number with short anal arms, with much inhibited oral lobes, and with gut development nearer the ventral surface than normal (Figs. 31, 32). Only a trace, or no, skeletal development was present. Many had short entodermal evaginations that showed little differentiation (Figs. 33, 34). Most had developed ventrodorsality, but this was not true of all (Fig. 34).

These experiments show that short time exposures of blastulae to weak solutions of thiourea have little effect other than inhibiting the invagination reaction (giving exogastrulae) in from 10 to 20 per cent of the cases. With increased concentrations and exposure periods, the degree of differential inhibition increases. This is particularly true as regards the inhibition of oral lobe, anal arms, and skeleton. There appeared to be little in the way of differential recovery.

DISCUSSION

This work has shown thiourea to be an effective agent in modifying development in the egg of *Dendraster*. It has also added to the already extensive evidence that certain modifications are nonspecific in nature (Child, 1941*a*). Not only may the modifications produced by thiourea be interpreted on the basis of quantitative physiological differentials in the developing egg, but they also give added proof of the general validity of the concept of graded activity as the primary developmental pattern or patterns. Thiourea inhibits to a greater extent those regions of the developing egg and early embryo that have been shown by numerous tests (dye reduction, indophenol blue reaction, etc.) to be the most physiologically active.

The types of modification produced by thiourea are not new but have been caused by a large variety (see Child, 1948, for references) of different agents (cyanide, azide, lithium, thiocyanate, pilocarpine, crowding, tobacco smoke, etc.). Under certain conditions all these agents will differentially inhibit the more active apical end of the polar gradient and the more active ventral region of the ventrodorsal gradient. To a certain extent all will inhibit the invagination process (causing exogastrulation) and under extreme inhibitory conditions lower the basal presumptive ectoderm to the physiological level of entoderm.

Many agents cause a decrease in scale of organization by differentially inhibiting the apical or ventral regions of the developing egg. Thiourea is quite effective in the inhibition of both these regions of the developing egg. With certain concentrations of thiourea (as well as with many other agents) the ventral area and oral lobe are both decreased in size, and the organization under the influence of these fields is diminished. This may be especially noticeable in the relationship of gut to ventral region. Up to a certain point, as differential inhibition increases, the invagination of the gut comes closer to (and more directed toward) the ventral side (Figs. 15, 16, 17, 22, 23, 27, 28, 31, 32). Data showing a decrease in scale of organization (through differential inhibition) or an increase of scale (through differential conditioning or recovery, as shown with many agents) offer excellent proof of the quantitative aspects of the early gradient patterns.

It should be pointed out, however, that all inhibitory agents do not behave exactly alike. Some readily cause exogastrulation, while others do not. In concentration gradients of certain enzyme inhibitors, Pease (1941, 1942*a*, *b*) showed that only certain of his inhibitors would cause a shift in the ventrodorsal axis of the developing *Dendraster* egg. Rulon (1946) showed that treatment of unfertilized eggs with NaCNS in Ca-free sea

water caused ectodermization, while treatment under similar conditions with LiCl caused entodermization. There are other examples. While it is fully recognized that the same agent may produce exactly opposite effects (through differential conditioning or recovery) by varying concentrations or exposure periods, it appears that different agents may, at least in some cases, have different effects because of differential action on certain of the underlying gradient correlates.

The phenomenon of exogastrulation may be a case in point. Sodium azide may, under favorable conditions, cause as high as 90 per cent exogastrulation (Rulon, 1949*b*). In these experiments with thiourea, only 20 per cent exogastrulation could be obtained. With maleic acid (Rulon, 1949*a*) no exogastrulae were found, although other types of inhibition were much in evidence. Among other actions it is well known that azide is an inhibitor of the type of oxidative metabolism associated with cytochrome oxidase. It appears from studies on the indophenol oxidase reaction in *Dendraster* (Child, 1941*b*) that cytochrome oxidase activity may in some way be associated with the invagination reaction. It is tempting to attribute the high percentage of exogastrulae caused by azide and certain other inhibitory agents as the result of inhibition (directly or indirectly) of cytochrome oxidase activity at the site of invagination as well as at the apical region. Yet cytochrome oxidase activity is certainly not the only activity involved in invagination. Phosphorylations appear to be highly important in amphibian gastrulation (Barth and Jaeger, 1947). This may well be the case in echinoderm gastrulation. Phosphorylations and numerous other biological processes are inhibited by azide. Thiourea may not have a direct effect on cytochrome oxidase, as found by Lerner and Chaikoff (1945) in thyroid tissue, yet it causes a certain number of larvae to exogastrulate. This may indicate that other oxidative enzymes are concerned with invagination. Maleic acid (an inhibitor of succinic dehydrogenase and glyoxalase) appears to have little effect on invagination, although it is a good inhibitor of other developmental phenomena. This may be because maleic acid has little or no effect on the underlying processes concerned with gastrulation.

At the present time it appears that, while many different processes underlie gastrulation and the inhibition of these processes, directly or indirectly and singly or collectively, may cause exogastrulation, the role of certain factors (cytochrome oxidase, other oxidative enzymes, phosphorylations, etc.) may be more important to the invagination reaction than others (succinic dehydrogenase, glyoxalase, etc.).

SUMMARY

1. Cleavage proceeded slowly and irregularly, but no differentiation occurred when the eggs of *Dendraster* were treated with certain high concentrations of thiourea in Ca-free sea water before fertilization.

2. Continuous exposure of newly fertilized eggs to low concentrations of thiourea gave slight inhibition of oral lobes and anal arms and caused approximately 20 per cent exogastrulae with differentiated ectoderms to occur. With higher concentrations the degree of inhibition increased, and exogastrulae appeared with inhibited ectoderms and enlarged entoderms.

3. Most larvae developed normally on return to sea water after 6–12 hours' exposure to low concentrations of thiourea immediately after fertilization. With slightly higher concentrations there was

some inhibition. Some exogastrulae with well-differentiated ectoderms and moderate-sized entoderms also appeared.

4. Continuous exposure of mid-blastulae to low concentrations of thiourea gave results similar to those found with continuous exposure beginning immediately after fertilization. Blastulae, however, tolerated high concentrations that were lethal to the newly fertilized egg. Development in these higher concentrations gave cell-filled, inhibited larvae which failed to develop anything in the way of skeleton. A number of entodermized exogastrulae also appeared if the concentration was not too high, but entoderm development, as well as bilaterality, was often inhibited at extreme concentrations.

5. Short time exposures of mid-blastulae to low concentrations of thiourea had little effect other than causing 10–20 per cent exogastrulation. With higher concentrations and longer exposure periods, inhibition appeared which approximated, but did not reach, that of continuous exposure.

6. A comparison of the action of thiourea, sodium azide, and maleic acid on sand-dollar exogastrulation is made, and a possible explanation of the differences in action of these agents, based on their different effects on enzyme activity, is given.

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HEATING RATE AND HEAT-SHORTENING TEMPERATURE IN EISENIA FOETIDA (SAVIGNY)[1]

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For the comparison of onset temperature of heat shortening of animal tissues, it is necessary to avoid introduction of any possible influence caused by the variations in heating rate. In an earlier paper published some twenty years ago, the media in which tissue was immersed were heated at the constant rate of about one degree (0°.97–1°.02 C.) per minute (Watanabe, 1930). The necessity of such precaution had already been pointed out by Engelmann (1874) and Ewald (1919*a*, *b*), and it had also been taken into consideration by careful workers (e.g., Simpson and Finney, 1930). According to Kühne (1864), the heat shortening will occur at different temperatures according as the tissue is rapidly or gradually subjected to the high temperature. However, it has not yet been made sufficiently clear how heating rate affects the heat-shortening temperature. For an analysis of heat-shortening phenomena, determination of the quantitative relation between heating rate and heat-shortening temperature should, theoretically, be of much interest. The present paper is primarily concerned with giving a simple, empirical formula, which expresses well the relation in *Eisenia foetida* to the constant rates of heating—rates easily obtained with a simple apparatus—ranging from about 0°.5 C. to 7° C. per minute. With use of numerical values for the constants in the formula thus obtained, which are independent of any particular rate of heating, an attempt was made to compare certain characteristics of body-wall tissues from different levels of the body of this animal.

MATERIAL AND METHODS

The worms were reared in wet horse manure from mid-autumn to early winter. The material used was all of mature form and was nearly the same in body-length (average, *ca.* 100 mm.) as well as in number of segments (average, *ca.* 100). The specimens obtained from the field in Hokkaido and from northern Honshu are usually smaller (cf. Sasaki, 1924). Latimer (1898), Vernon (1899), and Shafer (1928*a*, *b*) showed that a fatigued muscle shortened less and also at a lower temperature than a normal muscle did in heat shortening and that completely fatigued muscle did not show heat shortening. Hatai (1924) also pointed out that to employ fresh and active specimens was essential to the precise determination of the point of onset of heat shortening on gradual heating, since older and less active specimens shortened so gradually that determination of the point of onset with accuracy was difficult. These points were taken into consideration, and the material was used in as good condition as possible.

The technique employed in the present investigation is principally the same as that used in connection with the

[1] An abstract of this paper was read before the second annual meeting of the Hokkaido chapter of the Zoölogical Society of Japan, at Sapporo, November 12, 1949. The writer wishes to express his gratitude to Dr. C. M. Child and Dr. A. C. Giese, of Stanford University, for assistance in preparation of the manuscript.

writer's former work (Watanabe, 1930), but in this case the utmost care was taken, though the apparatus used was of a simple nature. A piece of the body wall was cut into a strip about 15 mm. long, nerve cord, alimentary canal, and main blood vessels being carefully removed, and was attached by S-shaped copper hooks at two points on the ventral median line 10 mm. away from each other. Then, before experimentation, pieces were kept in a moist chamber for $2\frac{1}{2}$ hours at a temperature of approximately 15° C. The writer's observations on CO_2 output in earthworms, *E.* (= *Allolobophora*) *foetida* (Watanabe, 1930) and *Pheretima hilgendorfi* (Watanabe, unpublished data), and in a polyclad, *Stylochus ijimai* (Watanabe and Child, 1933), show a marked increase in a single piece, and very wide variations in pieces from different levels of body occur following section; but, as the effect of section decreases, the rate of CO_2 output becomes constant, and a definite form of axial differential becomes evident 2–3 hours or more after section. The gush in CO_2 output and the variations may introduce variations in hydrion concentration in tissue and media. These conditions may not be favorable for an exact determination, since Ewald (1919*a*, *b*), Shafer (1928*a*, *b*), Simpson and Fenney (1930), and others have shown that the heat-shortening temperature of muscle and tendon varies according to hydrion concentration in the media.

A heating apparatus consisting of a 35-cc. thin-walled glass vial was suspended in a thermos bottle. The necessary temperature control was obtained by well-water in which the tissue was immersed. Electric power, with a current regulator, was supplied through nichrome wire surrounding the vial. For rapid equilibration of heat in the vial a small stirrer was used. Throughout this experiment heating was started at about 15° C. The tracings were recorded on smoked paper on a kymographic drum with use of a simple suspension method. The recording lever was perfectly balanced before measurement, and minimal tension was then put on the strip of tissue by advancing a light aluminum-wire rider. These precautions were taken because the loading was found to exercise some influence on the temperature at which the shortening took place.

A mercury thermometer used in this experiment was graduated from 0° to 50° C., with 0°.1 calibration; it was possible to estimate to 0°.01 C. Thermometric lag of this thermometer was determined by the method called "absolute determination" (Harper, 1912) and was found to be approximately 2 seconds, with the decimal value uncertain, but less than one-tenth second on the average. Correction of the thermometric reading was made in each case on the basis of this lag period and heating rate.

EXPERIMENTAL RESULTS

In general, the onset temperature of heat shortening became higher as the rate of heating increased. In order to obtain a quantitative picture of the general relation, the body-wall tissue from the anterior preclitellar region was heated at nine different rates, namely, at 0°.53, 0°.76, 1°.03, 1°.74, 2°.50, 3°.70, 4°.74, 5°.93, and 7°.78 C. per minute. For each rate of heating 50 worms were used.

Figure 1 gives the average data from the determinations. In the graph the circular outlines and the right-hand ordinates and upper abcissa scales show that heat-shortening temperature increases relatively more with increasing rate of heating up to about 4° per minute but that at the higher rates the increase is less and gradually tends to approach a certain limit. Plotting the pairs of recip-

rocal values ($1/x$ and $1/y$) and drawing the curve that would best describe the relation, we find a rectilinear function (solid circles and left-hand ordinates and lower abscissa scales in Fig. 1):

$$\frac{1}{y} = \frac{a}{x} + b \quad \text{or} \quad y = \frac{x}{a + bx},$$

in which x denotes the heating rate in degrees per minute, y the onset temperature in ° C., and a and b constants. With the use of least squares, we obtain $a = 0.000645$ and $b = 0.02361$. The probable errors of the equation and its two con-

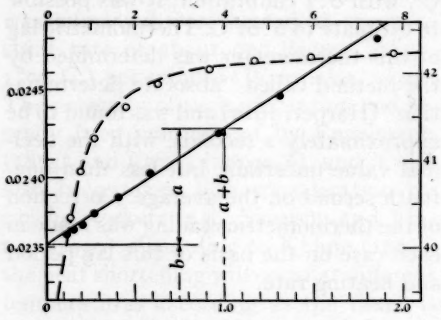

FIG. 1.—Effect of increase in heating rate upon onset temperature of heat shortening and linear relation between their reciprocals. Ordinates and abscissae are defined in the text.

stants are: $r_0 = 0.0382$, $r_a = 0.0000168$, and $r_b = 0.0000100$. Thus, when the heating rate is 1° per minute, the heat-shortening temperature to be expected is calculated from the equation as $1/(a + b) = 41°.22$ C. Similarly, when the heating rate approaches infinity, the heat-shortening temperature approaches $1/b$ —numerically, in this case, 42°.35 C. A rough approximation of these values can be obtained by a graph without difficulty, since $a + b$ represents the value for $1/y$ at $1/x = 1$ and b is equivalent to $1/y$-intercept of the line.

So far as the range of heating rate here applied is concerned, the formula sought holds fairly well; but it is still uncertain whether it will hold beyond this range. When the heating rate is much slower, the onset point of shortening cannot always be exactly determined, since the shortening often takes place too gradually to permit precise determination; moreover, with such a simple apparatus, it becomes rather difficult, with slower rise of temperature, to keep the rate of heating exactly constant. And, with too rapid a rise of temperature, the exact recording of temperature cannot be made on the drum, unless an adequate automatic recording method is devised.

Next, for purposes of comparison, another stock of body-wall strips from both ends of the body, anterior and posterior, and from the middle region of the same individuals was examined at five different rates of heating, ranging from 0°.45 to 6°.95 C. per minute. These tissue strips were of the same length and were examined in the same way as described above. The anterior ends of the middle strips were exactly at the fortieth segment.

Averages obtained from 35 worms for each rate of heating are presented in Figure 2, in which reciprocals of heat-shortening temperature in ° C. ($1/y$) are given as ordinates and those of rates of heating in minutes per degree ($1/x$) are given as abscissae. It is seen at once that the data disclose a linear relation of $1/y$ different $1/y$-intercepts for different to $1/x$, but with different slopes and body levels. The calculated values for a and b with their probable errors, those for $1/(a + b)$ and $1/b$ and for b/a, are given in Table 1.

The graphed data in Figure 2 show that at the same rate of heating the onset temperature of shortening is higher in the anterior than in the middle region and is again higher in the posterior region, though these values tend to show some approximation to each other with each

increase in rate of heating. Accordingly, the values for $1/(a+b)$ and for $1/b$ are also higher in both anterior and posterior regions than in the middle, since, as noted above, they correspond to values for the onset temperature on heating at the rate of 1° C. per minute and on heating as rapidly as possible, respectively. Thus, certain characteristics of heat shortening

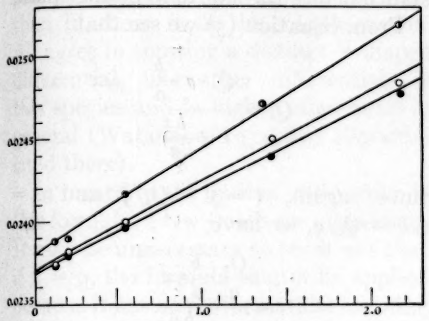

FIG. 2.—Reciprocal of onset temperature of heat shortening *vs.* reciprocal of heating rate for pieces from anterior (*A*), middle (*M*), and posterior (*P*) regions of the body. Ordinates and abscissae are defined in the text.

in the tissue may be said to show a distinct V-shaped differential along the axis of the body, independently of rate of heating.

GENERAL REMARKS

As the graphed data show, the onset temperature of heat shortening in body-wall tissue of *E. foetida* differs with rate of heating (broken line in Fig. 1). Through the limited number of points of observation described above many curves may in theory be drawn, but for the sake of practical convenience the following formula is chosen because of its simplicity in fitting and in defining the experimental meaning of the constants involved:

$$y = \frac{x}{a + bx} \qquad (1)$$

or

$$y = \frac{1}{\frac{a}{x} + b}, \qquad (2)$$

where x represents the rate of heating in degrees per minute, y, the onset temperature of shortening in ° C., and a and b, constants. From equation (2) we see that, when the tissue is continuously heated at the rate of 1° per minute, i.e., $x = 1$, the onset temperature will be $1/(a+b)$; and, when the heating rate approaches infinity and $1/x$ approaches 0, the maximal onset temperature is reached and approaches the asymptotic value of the equation, $1/b$.

TABLE 1

CALCULATED VALUES OF CONSTANTS a AND b

Parts	Anterior	Middle	Posterior
a	0.000502	0.000696	0.000432
r_a	0.0000123	0.0000193	0.0000158
b	0.02368	0.02375	0.02370
r_b	0.0000102	0.0000235	0.0000591
r_0	0.03818	0.02966	0.04283
$1/(a+b)$	41.36	40.92	41.27
$1/b$	42.23	42.10	42.19
b/a*	47.19	31.25	44.35

* Meaning of b/a will be discussed later.

The quotient of the two variables, x/y, indicates the velocity of the change in the tissue which gives rise to heat shortening, since y/x represents the time necessary for appearance of shortening on heating at the rate of x degrees per minute from 0° C.[2] This throws equation (1) into the form:

$$\frac{x}{y} = a + bx. \qquad (3)$$

If we put $1/(a+b) = m$ and $1/b = n$, we obtain

$$\frac{x}{y} = \left(\frac{1}{m} - \frac{1}{n}\right) + \frac{x}{n} = \frac{1}{m} + \frac{x-1}{n}.$$

[2] Strictly speaking, the biological zero point for the tissue activity should be taken as the initial temperature of the experiment, if it is known.

At any rate, it suggests that the velocity of this change in the tissue is a function of the first degree in rate of heating, x.

Rearranging the terms in equation (3), we find

$$\left(\frac{1}{y}-b\right)x=a. \qquad (4)$$

When we take q for the average heat capacity of the tissue substance and Q_s and Q_t for the heat quantity necessary for heat shortening at an infinitely high rate of heating and at the rate of heating x, respectively, then

$$q=\frac{Q_s}{s-t_0}=\frac{Q_t}{t-t_0},$$

in which temperatures are $s > t > t_0$.

Setting the initial temperature equal to 0° C., i.e., $t_0 = 0$,[2] we find

$$q=\frac{Q_s}{s}=\frac{Q_t}{t}.$$

Then,

$$s=\frac{1}{b}=\frac{Q_s}{q}$$

and

$$t=y=\frac{Q_t}{q}.$$

Using these terms, equation (4) can be written:

$$\left(\frac{q}{Q_t}-\frac{q}{Q_s}\right)x=a$$

or

$$(Q_s-Q_t)\frac{qx}{Q_sQ_t}=a,$$

which, by rearrangement, becomes:

$$(Q_s-Q_t)\frac{bx}{a}=Q_t. \qquad (5)$$

Equation (5) may be interpreted, if we assume that the heat quantity necessary for heat shortening at the rate x is proportional, first, to the heating rate, x, and, second, to the difference between the maximum attainable quantity of heat used at infinitely high rate of heating and that to be used at the heating rate x. If this interpretation is valid, then the constant b/a may indicate a utilization capacity, in the tissue, of heat, which is supplied to it from outside. In other words, b/a may give the relation between the fraction of heat utilized by the tissue for heat shortening and the total heat supplied to this system of tissue substance before the shortening takes place.

From equation (5) we see that

$$Q_s=\frac{Q_s\cdot x\cdot\frac{b}{a}}{1+\frac{bx}{a}}.$$

Since, again, $t = y = Q_t/q$ and $s = 1/b = Q_s/q$, we have

$$t=\frac{\frac{Q_s}{q}\cdot\frac{b}{a}\cdot x}{1+\frac{bx}{a}}.$$

Therefore,

$$y=\frac{\frac{1}{b}\cdot\frac{b}{a}\cdot x}{1+\frac{bx}{a}}. \qquad (6)$$

Equation (6) can also be derived directly from equation (1). This form of equation resembles fairly closely Langmuir's formula of the adsorption-isotherm, in which y corresponds to the amount of gas adsorbed on the surface at gas pressure x, $1/b$ corresponds to the saturated amount of adsorbed gas, and b/a is a factor equivalent to what is called the "adsorption coefficient" and is concerned with the activation energy of the reaction. The comparison of these two formulas suggests the important significance of the factor b/a. This factor can readily be found in equation (3), which is rewritten as follows:

$$\frac{x}{y}=a\left(1+\frac{bx}{a}\right).$$

That is to say, the velocity of the change caused by heating in the tissue is an increasing rectilinear relation to the b/a fraction of heating rate x. The calculated values for b/a are presented in the last line of Table 1.

For the different levels of the body the values for the constants, $1/(a+b)$, $1/b$, and b/a, are different and are higher in pieces from both extremities of body than in those from the middle region. All agree in showing a distinct V-shaped differential, like other differentials in this species and in higher oligochetes in general (Watanabe, 1931, and literature cited there).

In regard to the limit of application of this formula, a few words are added here. It may be unnecessary to point out that, if $x = 0$, the formula cannot be applied, because it has no mathematical meaning. In fact, when $x = 0$, no change occurs in temperature; thus heat shortening never takes place at the initial condition of temperature, no matter whether it is kept at 15° C. or at 0° C. As described above, when the heating rate was too high or too low, the onset point of shortening could not be determined with accuracy. Besides these technical difficulties, it must be kept in mind that heat shortening does not occur at all when the tissue is subjected to too low a temperature. In the present work this lower limit of temperature necessary for heat shortening was not studied, but, so far as the writer's experience is concerned, it does not occur in well-water in *E. foetida*, at least below 37° C.[3] If onset temperature is 37° C., the corresponding rates of heating to be expected, from this formula and with the values for constants given in the preceding table, are: 0°.15, 0°.22, and 0°.16 per minute for anterior, middle, and posterior pieces, respectively. Consequently, it may be seen that the formula does not hold, at least, for $x < 0.22$.

The temperature of the wet horse manure in which the worms were reared was often 38° or higher but produced no heat shortening in *Eisenia*. In this respect, however, it should be noted that the onset temperature of heat shortening may be altered by adding different amounts of various salts to the media in which the tissue is heated (Vernon, 1899; Moore, 1902; Meigs, 1919). In an earlier work it was found that onset temperature of heat shortening in *E.* (= *Allolobophora*) *foetida* and *A. caliginosa* became higher as the salt-content in water increased (Watanabe, 1930). It may be presumed that the water in the horse manure with which the worms were in contact contained salts or other substances which possibly prevented heat shortening to some extent.

[3] According to Hatai (1922), in this species the irritability apparently disappears at about 37° C., and the first heat rigor sets in at about 40° C. and culminates at about 43° C.

SUMMARY

The relationship between rate of heating and onset temperature of heat shortening of body-wall tissues from three different body levels in *E. foetida* has been studied. By increasing the rate of heating, the onset temperature shifts progressively to a maximum. The general relation can be expressed by an empirical formula:

$$y = \frac{x}{a + bx} \quad \text{or} \quad y = \frac{1}{\frac{a}{x} + b},$$

in which x is the rate of heating in degrees per minute, y is the onset temperature in ° C., and a and b are constants. All the data obtained can be fitted by the same formula, but values for the constants a and b are different for different levels of the body. The quantity $1/(a+b)$ is equivalent to the onset temperature

on heating at $x = 1$; $1/b$ is equal to maximum onset temperature obtained when x approaches infinity; and b/a is a sort of coefficient of utilization of heat for heat shortening of the tissue. The calculated values for $1/(a + b)$, $1/b$, and b/a are all higher in pieces from anterior and posterior regions than in those from the middle and show a shallow V-shaped form of axial differential which is characteristic of the species. Some analytical remarks on the formula are appended.

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THE COMPARATIVE HYPOXIC RESISTANCE OF HIBERNATORS AND NONHIBERNATORS

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MANY investigations have been made on the resistance of different species of animals to hypoxic conditions, which studies have included the adult, the young, the newborn, and even the unborn removed from the uterus or the marsupial pouch. The greater hypoxic resistance of the young appears to be due to such factors as incomplete homeothermism, cerebral metabolic rate, and glycolytic ability (Himwich, Fazekas, and Alexander, 1941; Himwich, Bernstein, Herrlich, Chesler, and Fazekas, 1942; Selle and

Witten, 1941; Selle, 1944; Hiestand *et al.*, 1944; Hiestand and Nelson, 1946). It has been definitely established that the newborn mammal has an additional source of energy (anaerobic) which is lacking in the adult (Reiss, 1931; Himwich *et al.*, 1941, 1942; Chesler and Himwich, 1944).

Various methods have been employed to subject animals to hypoxic conditions, including placing them in atmospheres of various percentages of oxygen and nitrogen, subjecting them to decompression, tying off the cerebral circulation, drowning, and determining survival time of the isolated head. The chemical nature of the survival energy has been examined by the use of enzyme inhibitors, such as cyanide, fluoride, iodoacetate, and by the use of glycotropic substances, such as adrenalin, insulin, etc. (Himwich *et al.*, 1941; Hiestand *et al.*, 1944).

In studying low-temperature adaptation in our laboratory, we have had occasion to note the apparent indifference to lowered oxygen pressures exhibited by the thirteen-lined ground squirrel during hibernation. The suspicion that the animal's ability to reduce its metabolism in hibernation might depend upon its ability to resist low oxygen tensions in the nonhibernating state led us to compare the hypoxic resistance of species capable of hibernating with other species which are known to be unable to hibernate. For this purpose we chose three species of mammals capable of hibernating, namely, the thirteen-lined ground squirrel, *Citellus tridecemlineatus;* the Syrian or golden hamster, *Cricetus auratus;* and the big brown bat, *Eptesicus fuscus*. The hypoxic resistance of these three species was compared with that of five species of nonhibernators, namely, the rat, mouse, guinea pig, and two species of birds, the pigeon and the English sparrow. Only adult animals were used. In all the experiments we purposely avoided using any animal in the hibernating state, since we were interested in the comparative (species) differences in hibernators and nonhibernators. Thus the differences in survival time are species differences and not differences in the physiological state of the individual. Had we used any animal during hibernation, we should have had to involve such factors as low body temperature. By the term "hibernator" we refer to the animal capable of hibernating but in the active nonhibernating state. The difference in survival time of the hibernator and nonhibernator species then becomes the more significant. Measurements of oxygen consumption during hibernation made in our laboratory have shown a tremendous reduction in metabolic activity. This is also indicated by the pulse rate, which may be as slow as five beats per minute in the ground squirrel, in which it is 200–400 beats per minute in the nonhibernating state (Johnson, 1928). Studies of the reduced state of metabolism during hibernation have been made by many (see Johnson, 1931).

MATERIAL AND METHODS

In order to produce an atmosphere of hypoxia, we used a decompression apparatus consisting of a vacuum pump attached to a large ballast jar, which, in turn, was connected to the animal jar by way of a large-bore stopcock. The animal jar was a bell jar with a volume about one-seventh that of the ballast jar. Between the two containers was placed an "inverted" Hg. pressure gauge, consisting of a small-bore glass tube connected at its top with the pressure line of the two chambers. The bottom end of the pressure gauge was fastened with rubber hose to a Hg. leveling bulb. Behind the glass tube was placed a millimeter scale, reading down from zero at the top to 780 mm. at the bottom. By

raising or lowering the leveling bulb, the Hg. level was adjusted to the barometer reading at the time of the experiment, and thus no other correction for standard barometric pressure was necessary. The air in the ballast jar was evacuated until a pressure of 25 mm. Hg. was reached. The animal was then placed in the bell jar and the stopcock opened. Within a second the ambient pressure surrounding the animal became reduced to 106 mm. Hg. (equivalent to an altitude of approximately 46,000 feet). The pO_2 of the ambient air in the animal chamber was thus explosively reduced to approximately 21 mm. Hg. or to the equivalent of 3 per cent oxygen at sea level. Simultaneously with turning the stopcock, a stopwatch was started, and the survival of the animal was determined, the end-point being the last respiratory movement.

TABLE 1

SURVIVAL TIME OF 5 SPECIES OF NONHIBERNATING AND 3 SPECIES OF HIBERNATING ANIMALS AT 106 MM. HG. ATMOSPHERIC PRESSURE (pO_2 21 MM.) PRODUCED BY SUDDEN DECOMPRESSION AT AMBIENT TEMPERATURES OF $24° \pm 2°$ C.

Species	No. of Animals	Av. Survival Time			Range
		Hr.	Min.	Sec.	
Sparrow	17	....	....	21	18″ –25″
Pigeon	3	....	....	48	41″ –60″
Mouse	16	....	....	28	18″ –37″
Rat	12	....	1	14	36″ –1′26″
Guinea pig	9	....	2	54	1′44″–3′46″
Hamster	18	....	8	54	2′9″–16′23″
Ground squirrel	12	....	18	3	3′30″–45′56″
Bat*	11	1	0	16	4′25″–3°+

* Some of the bats were used at different temperatures for comparative reasons. They were removed from the decompression chamber shortly after the final gasp and were revived, apparently none the worse for the experience. The above figures for bats are averages for all ambient temperatures from 10°.5 to 30° C. The individual bat records are shown in Table 2.

RESULTS

As can be seen from Table 1, considerable difference in time of survival occurred with the different species, especially between the groups of hibernators and nonhibernators. It should be mentioned also that the behavior of the two types of animals was quite different. The nonhibernators died quickly after convulsing, whereas the hibernators died slowly and more quietly. This was especially true of the bats and ground squirrels, which for some time at the low pressure appeared to breathe almost normally, sometimes for as long as 30–120 minutes. Respiratory distress was far less apparent in these forms. The bats appeared to succumb to hypoxia either within a few minutes (9 or less) or else were able to survive for over an hour. The type of breathing differed for these two periods, being a gasping (apneustic) type in the former and polypneic in the

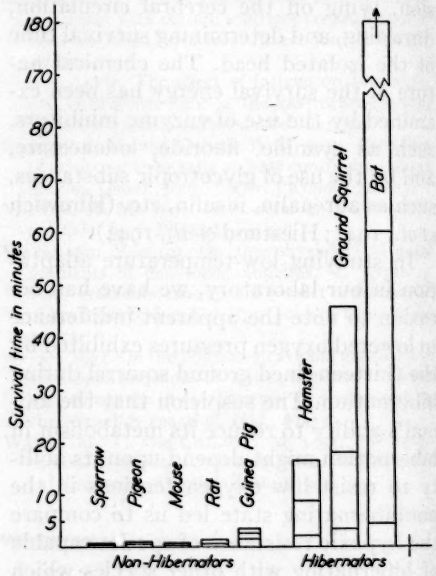

FIG. 1.—Graphic representation of hypoxic survival of the eight species used. The top horizontal line of each column represents the longest survival time of each group of animals; the middle horizontal line represents the average survival time of each group; the bottom horizontal line (when present) represents the shortest survival time of each group.

latter. We suspected that the difference might be a temperature effect and therefore exposed bats to different ambient temperatures, the results of which are shown in Table 2. As indicated by the table, some of the bats were used more than once. It was found possible to re-

TABLE 2

SURVIVAL TIME OF THE BAT AT DIFFERENT AMBIENT TEMPERATURES AT 106 MM. HG. ATMOSPHERIC PRESSURE PRODUCED BY SUDDEN DECOMPRESSION

BAT NO.	AMBIENT TEMP. (° C.)	SURVIVAL TIME		
		Hr.	Min.	Sec.
7	19.5	3	+	.....
1	19.5	2	0	30
1	21	2	29	30
5	23	.....	6	12
8	23	.....	49	55
7	25	1	47	43
2	25	1	12	.....
4	25	.....	9	4
3	26	.....	6	35
11	27	.....	4	25
9	30	.....	6	40
10	30	.....	5	54

move the bat from the decompression chamber after the last gasp and often to revive it by gentle massage. Bats revived in this manner were apparently unharmed by the previous decompression and showed no acquired hypoxic tolerance.

DISCUSSION

These findings suggest a correlation between an animal's ability to hibernate and its ability to withstand hypoxic conditions. Indeed, the ease with which a species can be made to hibernate parallels its degree of hypoxic resistance. Thus the hamster, which hibernates only if kept in the cold for many weeks, has the poorest anoxic resistance of the three species of hibernators, while the bat, which hibernates with simply a drop in the ambient temperature, has by far the greatest anoxic resistance. The ground squirrel lies between the bat and the hamster in both these respects. The bat's extreme hypoxic resistance was noted by Britton and Kline (1945), who reported survival of bats averaging 30 hours at 320 mm. Hg., a pO_2 considerably greater than that used in the present communication. In their work the bat was compared to the rat, which survived 2.5 hours at 320 mm. Hg. pressure. The difference in these two species is far more dramatic at lower oxygen pressures. Survival of the duck and chimney swift was found to be 6 and 8 hours, respectively, at 320 mm. pressure (Britton and Kline).

Probably as significant as anoxic resistance is the effect of temperature, both ambient and body temperature. Studying the effect of external temperature on hypoxic resistance as measured by simulated altitude and survival, several authors (Hiestand and Miller, 1944; Kottke, Phalen, Taylor, Visscher, and Evans, 1948) have found that higher altitudes can be reached by lowering the ambient temperature. It has been stated (Kottke *et al.*, 1948) that the survival ceiling for mice is increased approximately 1,000 feet per 1 degree drop in external temperature. From another aspect it has been suggested (Salhanick and Starr, 1949) that anoxic resistance increases an animal's resistance to cold, which partly accounts for the ability of the newborn (rat) to survive at low temperatures. Also it has been shown (Van Middlesworth, 1949) that rats exposed to anoxic conditions at low temperatures for 2 or more hours can then withstand severe anoxia for more than forty times as long as control animals can. This observation agrees with our findings with bats exposed to cold.

Delsaux as early as 1887 asphyxiated bats by placing them in a chamber the

air of which was rarefied by a vacuum pump to 50 mm. Hg. pressure. He noted that they could remain for half an hour at this pressure and still survive when removed. Saisy (1815) stated that hibernating animals can use all the oxygen in the chamber before death, while the rabbit can use only 75 per cent, the rat 62.5 per cent, and the sparrow 42.5 per cent. The great anoxic resistance of the bat is also shown by its survival under water (Johnson, 1931), which may be as long as 16 minutes. This is even exceeded by the hedgehog, which has survived 22.5 minutes under water.

SUMMARY

A comparison has been made of the anoxic resistance of three species of hibernators with that of five species of nonhibernators by subjecting them to a barometric pressure of 106 mm. Hg. (pO_2 of 21 mm. Hg.) explosively produced. At this pressure the survival was considerably longer for the hibernators. The greatest survival was shown by the big brown bat, one of which lived more than 3 hours. Next in order was the ground squirrel, and then the hamster. The shortest survivor was the English sparrow, which lived only 21 seconds. It is suggested that the ease with which a hibernator can be induced to go into the hibernating state parallels its hypoxic resistance. Much less respiratory distress is shown by the hibernators under hypoxic stress than by the nonhibernators in identical conditions. It is suggested that good anoxic resistance is a prerequisite to ability to hibernate.

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